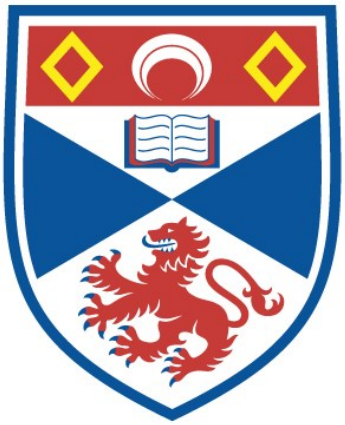
FERMENTATION SYSTEMS FOR ENHANCEMENT OF ETHANOL PRODUCTIVITY IN SACCHAROMYCES CEREVISIAE AT ELEVATED TEMPERATURES

Armando Marsden Lacerda Filho

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A Thesis presented by
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to the University of St. Andrews
in application for the degree of
Doctor of Philosophy.

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DECLARATION

I hereby declare that this thesis is based on work carried out by me, that the thesis is of my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, University of St. Andrews, under the supervision of Dr. William M. Ledingham.

Signature

Date 24.07.95

CERTIFICATE

I hereby certify that Armando Marsden Lacerda Filho has spent nine terms engaged in research work under my direction and that he has fulfilled the conditions of Ordinance General n°. 12 of the resolution of the University Court 1967, n°. 1, and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Signature of Supervisor Date

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ACADEMIC RECORD

I graduated with degree of Bachelor in Biomedical Science in 1974 from the Federal University of Pernambuco, Recife - Brasil.

I graduated with degree of Master in Biochemistry in 1987 from the Biochemistry Department of the Federal University of Pernambuco, Recife - Brasil.

I matriculated as a research student in the Department of Biochemistry and Microbiology, University of St. Andrews, in April 1988.

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I am also grateful to " The Council to Improve the Training of University Teachers " - CAPES and to the Federal University of Pernambuco, specially to the Micology Department for financial support and incentive.

I should like to express my gratitude to all members of my family, specially Virginia, Paula and Armando Neto. Many thanks to all the colleagues and friends which made my life in St. Andrews more enjoyable.

I would like to thank to Mr. Armitt and Mr. Hunter for their technical assistance and Mr. Leather for his help during these years, as well as all the Staff of the Biochemistry Department are also gratefully acknowledged.

ABBREVIATIONS .

β	concentration factor
^{o}C	degree centigrade
cm	centimeter
Co.	Company
D	dilution rate
Dm	maximum dilution rate
Dc	critical dilution rate
e.g.	exempli gratia (for exemple)
et al.	et alia (and others)
F	flow rate
g	gram
g/l	gram per litre
GLC	Gas Liquid Chromatography
h	hours
i.e.	id est (that is)
Inc.	Incorporation
K_s	saturation constant
L	litre
ln	natural logarithm
LTD.	Limited
M	molar
m	maintenance energy
mg	milligram
mL	millitre
min	minutes

μ	specific growth rate
μ_{max}	maximum specific growth rate
µg	microgram
µl	microlitre
nm	nanometer
p	product concentration
rpm	revolutions per minute
S	substrate concentration
S_o	substrate concentration at feed medium or limiting substrate concentration
S_r	residual substrate
Sigma	Sigma Chemical Co. Ltd.
t	time
t_d	doubling time
U	units
V	culture volume
v/v	volume per volume
x	biomass concentration
x_o	biomass concentration at zero time
x_{max}	maximum biomass concentration
Y	yield coefficient
$Y_{x/s}$	growth yield
$Y_{p/x}$	product yield

S U M M A R Y

Three Brazilian yeast strains, *Saccharomyces cerevisiae* 42 - F, *Saccharomyces cerevisiae PLA 851 and Saccharomyces boulardii* IZ 1904, all currently employed in the sugar fermentation industry, were evaluated with respect to their thermal tolerance and alcohol production kinetics. Best performance was found in *Saccharomyces cerevisiae* PLA 851 at temperatures up to 40 degrees (a common fermentation temperature in the Brazilian industry). This strain was further evaluated in chemostatic growth under sucrose limitation with biomass feedback on a 1 Liter scale in a specially constructed apparatus.

At 30 degrees and 35 degrees under a dilution (growth) rate of 0.1 /h ethanol productivity increased by a factor of 2 with feedback and at 40 degrees by a factor of 3. The feedback factor (Beta) was 0.9. PLA 851 cells , heat - shocked at 45 degrees, resulted in a greater biomass productivity subsequently at 40 degrees coupled with a change in cell morphology.

Highest ethanol productivity was found with 10 % initial sucrose concentration at a dilution rate of 0,25 /h with feedback. Saccharomyces cerevisiae PLA 851 appears to be well adapted to the harsh physiological conditons in alcohol fermentations as currently practiced in Brazil.

CONTENTS

1 - INTRODUCTION

1.1 - ETHANOL AS A FUEL

Ethanol has been produced by traditional methods throughout the world for millenia as a beverage and preservative.

These ancient methods of production have remained almost unchanged and it is only recently with considerable interest in fuel ethanol that the fermentation processes are being looked at with a view to increasing production of fuel ethanol.

There are many factors related with ethanol fermentation, but we must take into account that three important areas of interest regarding the efficiency of fermentation process are: The choice of raw material, the choice of microorganism and the design of the fermenter.

The choice of a raw material depends upon which substrate is available, its price and its requirement for human or animal consumption.

This may bring in an element of competition between fuel for people or fuel for automobile engine as is the case for sugar cane and mandioca (cassava) in Brazil.

The second and third areas are very close regarding that the fermenter design may be modified to optimise conditions such as aeration, pH, temperature and the level of certain nutrients to the requirements of the microorganisms for optimal ethanol production.

However, the converse is also true to a considerable extent, that is, the microorganism may be genetically manipulated or selected naturally due to its ability to withstand or even thrive and ferment better in a pre-existing condition within the fermenter[1].

The raw materials currently in use in the United State for the production of ethanol by fermentation are numerous. Although corn (maize) is the most commonly used, others include milo (sorghum - grain), barley, potato - processing wastes, brewery wastes, soft - drink wastes, sugar cane molasses, citrus molasses, cheese, whey, confectionary wastes and used sulphite liquor from paper manufacturing[2,3,4].

There is a current interest, however in the development of more sugar - intensive substrate such as sweet sorghum, inulin - rich Jerusalem artichoke and of agricultural wastes such as corn stover[5].

1.1.1 - THE BRAZILIAN NATIONAL ALCOHOL PROGRAM

The Brazilian National alcohol program aimed at the replacement of petroleum with fuels(notably ethanol) derived from plant biomass, has been amongst the most ambitious and controversial energy-related projects to be undertaken by any developing nation.

For Brazil, the substitution of imported petroleum with biomass-derived fuel alcohol is made possible and greatly favoured by the vastness of the country and its tropical location with favorably conditions for production of biomass.

In order to be useful for large-scale production of industrial ethanol, feedstock must be available in sufficient quantity, at right cost and for a large part of the year and a commercial process for its utilization must be available.

Sugar cane is currently the main feedstock for the production of fermentation ethanol. It offers a potential yield of over 4,000 liters of ethanol per hectare per year, the highest yield for any crop, and the energy balance for ethanol production is more favorably than with other crops due to the contribution obtainable by burning the bagasse, the residue remaining after extraction of sugar [6].

Given the fact that the most of the biomass resources are found in the developing countries (122 countries in Asia, Africa and South America) all of which possess economies with are basically agrarian, the potential application and availability of these resources constitutes an important catalyst for economic and technological progress.

Indeed, many developing countries with agrarian-based economies differ from each other in terms of cultural systems, technological growth, society and government as well as in their approaches to the pressing problem of poverty, overpopulation and underdevelopment[7].

1.1.2 - HISTORICAL

For a nation such as Brazil, which until recently produced little petroleum, the use of alcohol as a petroleum substitute is not new.

The first commercial fuel based on ethanol was manufactured in 1927. In 1931, a government decree required that all gasoline contain at least 5 % ethanol in order to increase demand for sugar cane in the face of a worldwide depression. Alcohol was of little importance as a energy source until 1956, when the government decide to pursue an industrialization strategy based on automobile industry.

The consumption of oil in Brazil increased sixteenfold between 1946 and 1974, consequently the oil import bill had grown to 39.5 % of total exports. However, the fuel crisis of 1973, in which the price of crude oil quadrupled, prompted many countries to take stock of the energy options available to them, and in many cases, develop schemes for the production of energy from internally-produced renewable biomass.

In this context, the Brazilian National Alcohol Program (PROALCOOL), was established in 1975, with the goals of reduced expenditure on petroleum imports, diminishing individual and regional income inequalities, increasing national output by greater employment of casual labour (so-called "boias frias") and expanding

the production of capital goods by stimulating the distillery industry. The program aimed to produce 3 billion liters of alcohol annually by 1980. To achieve that production, the government promised a financial program, guaranteed acquisition of alcohol at fixed prices up to the quotas established by the Federal Institute of Sugar and Alcohol (I.A.A.) and an array of financial incentives.

The results exceeded expectations, in part because a sharp fall in the sugar world prices stimulated Brazil to divert raw cane from sugar refineries to alcohol distilleries. Alcohol production from 1979 - 1980 sugar harvest was 3.4 billion liters, 13 % over the goal and nearly six times the production achieved only 5 years earlier.

In 1979, due to the success of the program, the government of General Joao Figueiredo decided to initiate a second phase aiming to produce 10.7 billion liters of alcohol a year by 1985 and ordering commercial production of vehicles running on pure alcohol.

The production in the year 1982 - 1983 was 5.8 billion liters, with a saving of $ 1.3 billion in foreign exchange. In 1983 a third phase of the program was approved by the government with the aims of producing 14.3 billion liters by 1987 - 1988.

However, in 1985 the production of ethanol was about 11.9 billion liters a quantity well above expectations. In 1986 Brazil was producing about 30% more alcohol than it needed[8].

Nevertheless, the sharp fall in the world petroleum prices in that same year clearly made fuel alcohol less competitive with gasoline and there seems no immediate prospect that oil prices will rise sufficiently to make alcohol viable as a nonsubsidised source of fuel energy.

Although in 1991, a new event such as the Gulf war, has raised governmental awareness in many developing countries, particularly Brazil, of the important role that biomass for the production of ethanol as a fuel and chemical feedstock can play for the present and the future development of the country.

An interesting aspect about the Brazilian National Alcohol Program (PROALCOOL) is its focus on a specific sector; The sugar complex. In Brazil the sugar industry suffers from two important Achilles's heels - economic and political. Economically, it is sustained on the basis of government subsidies, which are increasingly difficult to maintain as the price of petroleum falls. Politically, the research program depends on the Ministry of Industry and Commerce, and has never been viewed favorably by Petrobras (The State Petroleum Company).

The complexity of the interests tied to biotechnologies are reflected in the difficulty of defining a strategy for the sector and by institutional fragility of the executive organs of research responsible for biotechnology in Brazil[9].

1.1.3 - DEVELOPMENT OF THE BRAZILIAN ALCOHOL PROGRAM

1.1.3.1.- GENERAL

Brazil has based its economic development primarily on imported petroleum and petroleum based technologies. The fuel crisis in 1973, in which the price of crude oil almost quadrupled, prompted many countries to take stock of the energy available to them, and in many cases, to develop schemes for the production of energy from internally-produced renewable biomass. The main reason for the creation of the Brazilian National Alcohol Program (PROALCOOL) was economic.

The program mainly aimed to reduce the country's import expenditure for petroleum, which in 1980 was estimated in US$ 11 billion. The wisdom of the program, now in its sixteenth year, continues to be debated in terms of its costs and benefits.

Some critics of the program claim that the growth of The PROALCOOL program has replaced petroleum supply risk with a new risk of disruption of alcohol supplies due to the growing number of alcohol-fueled cars as well as the geographic concentration of alcohol production, for instance, in 1983-1984 the State of Sao Paulo was responsible for 69 % of all ethanol produced in Brazil.

Some factors that could increase the risk of interruptions in ethanol supply, apart from the increasing number of alcohol-fueled cars nationally, are the vulnerability of the sugar cane crops to weather, pests, and diseases.

However, in the few years of PROALCOOL's existence there have been major productivity improvements in agriculture such as improved crop varieties, more intensive cultivation methods and better management and coordination of planting and harvesting.

Nevertheless, the technological process for ethanol production from sugar cane or molasses still needs improvement.

It is however necessary to emphasize that considerable advances in terms of research were made, particularly by Professor Walter Borzani and co-workers in the University of Sao Paulo - Brazil.

Indeed, improvements in fermentation technology as a strategy for reducing the cost of microbial chemicals production still remains a matter of priority.

Thus as long as the uncertainties associated with price and supply of petroleum continues, it can be expected that both new and conventional biological routes to chemicals from biomass will continue to be explored by industry.

1.1.3.2 - ADVANCES IN FERMENTATION TECHNOLOGY

Advances in fermentation processes as shown in TABLE 1, have already been implemented to some degree by industry, are under development, or considered to be directions for future development.

The majority of advances are directed toward improvements in product recovery, thus immobilizing cells, in effect, eliminates a cell-broth separation step; a higher operating temperature aids in the recovery of volatile products like ethanol; Cell recycle system and higher product tolerance cells enhance product concentration and recovery operation.

The utilisation of a highly efficient system is itself an important goal, mainly when associated with renewable source of energy, with low environmental impact and many positive points in social and development aspects[10].

TABLE 1 : ADVANCES IN FERMENTATION TECHNOLOGY

CONVENTIONAL	ADVANCED
BATCH FERMENTATION	CONTINUOUS, SEMI- CONTINUOUS
CELLS IN SUSPENSION	IMMOBILIZED CELLS
LOWER FERMENT. TEMPERATURE	HIGHER FERMENT. TEMPERATURE
CELLS USED ONCE	CELL RECYCLE / REUSE
NATURAL STRAINS	ENHANCED OR ENGINEERED STRAINS
DISTILLATION	LIQUID-LIQUID EXTRACTION, MEMBRANES, ADSORBENTS, IMPROVED DISTILLATION.
INDIRECT PRODUCT SENSORS	DIRECT PRODUCT SENSORS
SIMPLE CONTROLS	COMPUTER CONTROL.

1.1.3.3 - FUTURE PROSPECTS FOR THE PROALCOOL PROGRAM

PROALCOOL, the world's largest renewable fuel program, has a rather uncertain future. At the present, the program produces fuel alcohol to power some 30 % of Brasil's car who run on hydrated alcohol (96 % alcohol with 4 % water). Petrol,used in the remaining 70 % of car contains 20 - 22 % anhydrous alcohol blended in. The problem facing the program divide into 3 categories:

(i) Technical ones : these related essentially to the technical performance of alcohol engines and to corrosion. Despite modifications to fuel piping, tanks and carburetors (plating with such metals as zinc, tin and chromium) corrosion problems remain and frequent servicing is essential. This is particularly true of engines from the era 1982 - 1986. More modern engines offer better performance.

(ii) Economic ones : With a petroleum barrel price anywhere less than 60 US$, alcohol is more expensive to produce. With the present low price for world petroleum (US$ 18/barrel) a heavy subsidy is necessary to maintain the price differential of alcohol at the pump being 30 % cheaper than petrol. In Brazil, petrol users subsidise alcohol users. As alcohol production could easily be made more efficient, this leads to the third and possibly most serious problem.

(iii) Political : The State Petroleum Company, Petrobras, which is responsible for alcohol distribution in the whole of Brazil, is known to hold strong reservations regarding the continuation of the Proalcool program (It has probably always been politically against the program).

Petrobras may well have been responsible for many "difficulties" in distribution in past years which resulted in emergency importations of methanol and European wine-based spirits in the period 89/90 (though these were in part due to diversion of cane juice to sugar production rather than alcohol production in response to favorable world sugar prices).

The Government is funding the development of bus and lorry engines (to use alcohol) based on aeroengine technology. Previous attempts at development of biodiesel fuels have not met with success.

Unfortunately alcohol production is in the hands of a powerful class (Usineiros or Sugar Barons) with a great political influence, who have every reason to believe that, as in the past, they will be able to persuade the government, mainly for social reasons, to subsidise their inefficient industry.

1.2 - MICROBIAL GROWTH THEORY

1.2.1 - GENERAL PRINCIPLE

The object of quantitative theory is to predict the values of the growth rate and the concentration of biomass and substrate under different conditions. A batch culture is an example of a closed culture system which contains an initial, limited amount of nutrient.

The inoculated culture will pass through a number of phases (see Fig.1.2.1.). These phases reflect changes in the biomass and its environment. The exponential phase can be described by the equation:

$$dx/dt = \mu x \qquad (1)$$

where x is the concentration of microbial biomass, t is the time (h) and μ is the specific growth rate, in $hour^{-1}$.

On integration, equation (1) gives:

$$x_t = x_o.e^{\mu t} \qquad (2)$$

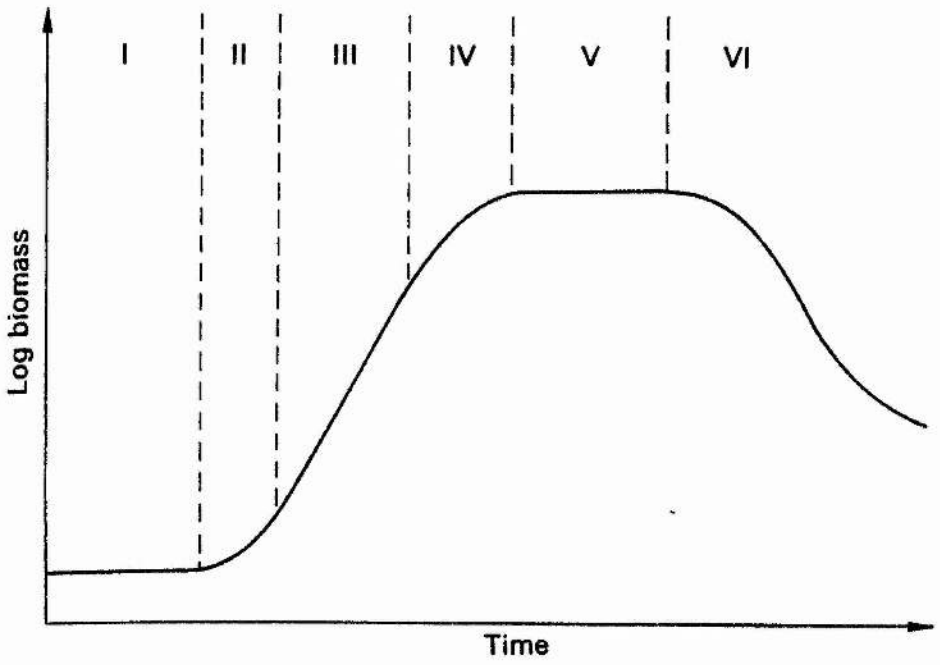


Figure 1.2.1 - Batch growth curve with 6 phases: I) Lag; II) acceleration growth; III) exponential growth; IV) decelerating growth; V) stationary and VI) decline.

Where x_o is the original biomass concentration, x_t is the biomass concentration after the time interval, t (h), and e is the base of the natural logarithm. On taking natural logarithms, equation (2) becomes;

$$\ln x_t = \ln x_o + \mu t \qquad (3)$$

Thus a plot of natural logarithm of biomass concentration against time should yield a straight line, the slope of which should equal μ.

During the exponential phase the organism is growing at its maximum specific growth rate (μ_{max}) for the prevailing conditions and equation (2) predicts that the growth will continue indefinitely.

However, growth leads to the consumption of nutrients and the excretion of microbial products; events which influence the growth of the organism. Thus, after a certain time, the growth rate of the culture decreases until growth finally ceases.

The nature of limitation of growth may be explored by growing the organism in the presence of a range of substrate concentrations.

The decrease in growth rate and the cessation of growth, due to depletion of substrate, may be described by relationship between μ and the residual growth-limiting substrate represented in equation (4) and (3).

$$\mu = (\mu_{max}.S)/ (K_s + S) \qquad (4)$$

Where S is the residual substrate concentration, K_s is the saturation constant, numerically equal to substrate concentration when μ is half μ_{max} and is a measure of the affinity of organism for its substrate[11].

The kinetics of product formation by microbial cultures in terms of growth-linked products and non-growth linked products has been discussed by Pirt[12].

The growth-linked products may be considered equivalent to primary metabolites which are synthesized by growing cells and can be described by the equation:

$$dp/dt = q_p.x \qquad (5)$$

Where p is the concentration of product and q_p is the specific rate of product formation. Also, the product formation is related to biomass production by equation:

$$dp/dx = Y_{p/x} \qquad (6)$$

Where $Y_{p/x}$ is the yield of product in terms of substrate consumed.

Multiplying the equation (6) by dx/dt gives:

$$dp/dt = Y_{p/x}.dx/dt \qquad (7)$$

but substituting the term $dx/dt = \mu x$ gives:

$$dp/dt = Y_{p/x} .\mu x \qquad (8)$$

combining equations (5) and (8) will have;

$$q_p = Y_{p/x} .\mu \qquad (9)$$

From the equation (9) it may be seen that when product formation is growth associated the specific rate of product increase with the specific growth rate.

1.2.2. - CHEMOSTAT THEORY

A culture of microbes can be prolonged by continuous addition of fresh medium. The continuous harvesting of any product can be increased by means of that system.

A chemostat culture should consist of a perfectly mixed suspension of biomass into which medium is fed at a constant rate and the culture is harvested at the same rate so that the culture volume remains constant.

After the formulation of the basic theory by Monod[13] and Novick & Szilard[14], the chemostat culture increased the perspective of a higher productivity in the fermentation processes.

According to Pirt [12], the three unique purposes of chemostat culture in the control of growth and behaviour of microorganisms are:

(1) The chemostat permits biomass growth rate to be varied with no change in environment other than the concentration of growth-limiting substrate. In a simple batch culture, growth rate changes can be only achieved by qualitative changes in nutrition or quantitative change in physicochemical conditions such as temperature or pH value.

(2) The second purpose is the converse of (i), that is to fix the growth rate while the environment is altered. This allows distinction between the effects of growth rate change and environment change.

(3) The third purpose is to keep substrate-limited growth with a constant growth rate. Substrate-limited growth can be obtained only transiently in a batch culture and it is always accompanied by changing growth rate.

This function of the chemostat widens the possible range of constant environments to include not only the extremes of excess and exhaustion of growth-limiting substrate but also all the intermediate states.

Essentially the chemostat method simplifies the culture system and thereby facilitates the elucidation of the reaction of the organism to its environment, and the control of microbial processes.

The advantages of this simplification are enhanced in importance when interactions of two or more species in a culture are to be investigated or controlled.

1.2.3 - CONTINUOUS CULTURE

Exponential growth in batch culture may be prolonged by addition of fresh medium to the vessel (see Fig 1.2.3). The flow of medium into the vessel is related to the volume of the vessel by the term dilution rate, D defined as D = F/V, where F is the flow rate and V is the volume, thus D is expressed in the units of reciprocal time (h^{-1}).

The net change in cell concentration over a time period may be expressed as:

$$dx/dt = (\mu - D)x \qquad (10)$$

Under steady-state conditions the cell concentration remains constant, thus dx/dt = 0 and u = D. Thus under steady-state conditions the specific growth rate is controlled by the dilution rate.

Substituting $\mu = (\mu_{max}.S)/(K_s + S)$ into equation (10), then

$$dx/dt = x \left\{ (\mu_{max}.S)/(K_s + S) - D \right\} \qquad (11)$$

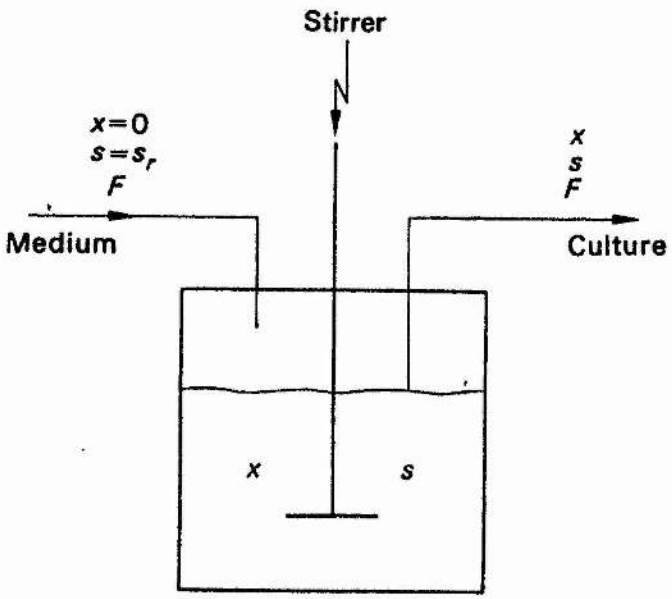


FIG 1.2.3 - Diagrammatic representation of the chemostat. The biomass and growth-limiting substrate concentrations at different points are represented by x and s respectively; F is the flow rate and V is the culture volume.

The net change in the residual growth limiting substrate concentration may be described by the equation below;

$$ds/dt = DS_r - DS - \mu_{max}.x/Y \left(S/K_s + S \right) \qquad (12)$$

At steady-state both ds/dt and dx/dt equal zero, then the value for x and S in steady-state are given by the expression:

$$\bar{x} = Y \left(S_r - S \right) \qquad (13)$$

and

$$\bar{S} = \left(K_s.D \right)/\left(\mu_{max} - D \right) \qquad (14)$$

Where x is the steady-state cell concentration and S is the steady-state residual substrate concentration.

The equation (14) explain the mechanism whereby D controls u.

Cell growth will result in the depletion of substrate until residual substrate concentration equals the substrate concentration which will support the growth rate equal to the dilution rate.

If the substrate is depleted below the level that will support the relevant growth rate, cells will be washed out at a rate greater than they are being produced and S will increase resulting in an increase in the growth rate and the balance would be restored. Thus, the system is a self-balancing one.

The maximum growth rate is obtained when $S = S_r$. Inserting this value in equation (4) we have

$$\mu = D_c = \mu_{max} \cdot S_r / (S_r + K_s) \qquad (15)$$

Where D_c is the critical dilution rate, at which value the steady-state biomass concentration is zero.

If $S_r >> K_s$ it follows from equation (15) that $D_c = \mu_{max}$.

When $s >> K_s$ we can put $\mu = \mu_{max}$ in equation (10) and on integration obtain

$$\ln x = (\mu_{max} - D) t + \ln x_o \qquad (16)$$

If we make $D > D_c$ in the chemostat, the culture biomass decreases, or washout occurs according to equation (16) and the slope of logarithmic plot is $(\mu_{max} - D)$, which gives the value of μ_{max}.

For the chemostat culture the rate of output of biomass per unit of culture is given by $R = Dx$ and in steady-state we have

$$R = DY \left\{ S_r - K_s D/(\mu_{max} - D) \right\} \qquad (17)$$

The biomass output rate reaches a maximum at dilution rate D_m, which is obtained by differentiating R with respect to D and equating the derivative to zero. Thus we find that;

$$D_m = \mu_{max} \left\{ 1 - (K_s/ S_r + K_s)^{1/2} \right\} \qquad (18)$$

Substituting in equation (13), we obtain for the steady-state biomass at D_m,

$$x_m = Y \left[S_r + K_s - \left\{ K_s(S_r + K_s) \right\} \right]^{1/2} \qquad (19)$$

If $S_r >> K_s$ then for the maximum output rate we have

$$D_m x_m \fallingdotseq D_m Y S_r \qquad (20)$$

1.2.4. - THEORY OF CONTINUOUS CULTURE WITH FEEDBACK OF BIOMASS

A chemostat fitted with some device to increase the biomass concentration above values possible in the simple chemostat, that is Y (Sr - S), is termed a chemostat with feedback of biomass, Herbert[15]; Pirt & Kurowski[16].

According to Pirt[12], the concentration of biomass may be achieved in various ways (see Fig.1.2.4; (a), (b), (c), (d)).

In system (a) a cell-free or dilute biomass stream is removed by filtration and there is also an outlet for the concentrated biomass suspension. In system (b) the culture is divided into two zones, an agitated homogeneous zone at the base where the growth occurs and, above the baffle plate , a sedimentation zone, virtually without growth, in which the biomass can sediment and return to the agitated zone. Thus a cell-free or dilute stream of biomass leaves the top of the fermenter and the concentrated biomass suspension leaves from an outlet in te growth zone.

In the "monostream" system (c) it is assumed that the biomass is concentrated by filtation or sedimentation in the outlet stream and ther is no outlet for the concentrated biomass.

In system (d) the biomass is concentrated outside the fermenter and the culture is separated into two streams of biomass one dilute and one concentrated; a part of concentrated stream is fed back to the fermenter.

The systems (a), (b) and (c) are represented by internal feedback model and system (d) by the external feedback model. We will consider the model depicted in Fig.1.2.4 (d) due to its direct relationship with our work.

In system (d) the effluent culture is passed through a separator, for example a centrifuge, which concentrates the biomass and produces both dilute and concentrated streams of biomass. Part of the concentrated stream is fed back to the culture vessel. The overall dilution rate is given by $F/V = D$ where V is the culture volume. The culture outflow rate is given by the equation below, where a is the fraction of the outflow liquid stream which is fedback.

$$F_s = F + aF_s \qquad (21)$$

From equation (21) it follows that

$$F_s = aF/(1-a) \qquad (21a)$$

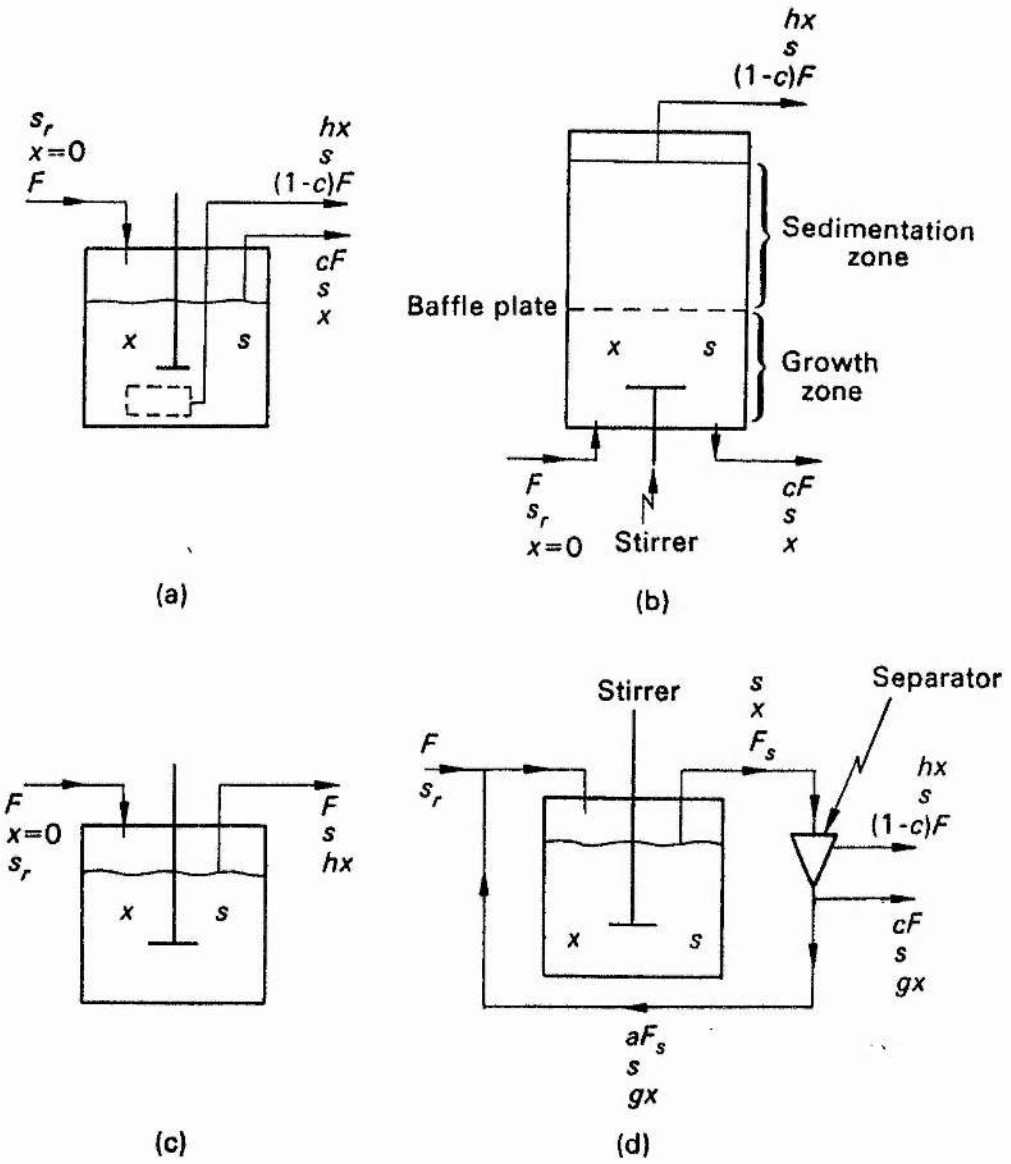


FIG 1.2.4 - Various systems for feedback of biomass in a chemostat: (a) internal filtration; (b) internal sedimentation; (c) monostream feedback; (d) external feedback. Symbols: F and F_s are flow rates; x is biomass concentration; s and s_r are growth - limiting substrate concentrations and c, g and h are constants dimensionless.

The separator concentrates the biomass by the factor g and since the amount of biomass fed back is agF_s, it can be seen that ag is the fraction of biomass leaving the fermenter, which is fed back. The biomass balance for the culture is given by

Net growth = growth - output + feedback

which for the whole culture will be

$$V.dx = V\mu x.dt - F_s x.dt + aF_s gx.dt \qquad (22)$$

substituting for F_s (Eq.21a) and dividing through by V.dt, equation (22) becomes

$$dx/dt = \mu x - Dx/(\ 1-a\ ) + agDx/(\ 1-a\ ) \qquad (23)$$

and in steady-state when dx/dt = 0 we have

$$(\ \mu - \beta D\ )x = 0 \qquad (24)$$

where $\beta = (\ 1-ag\ )/(\ 1-a\ )$

The factor β is a positive fraction because $1 > ag > a$.

If ag = 1, it would mean that all the biomass is fed back and there could not be a steady-state. In the steady-state, $\mu = \beta D$, thus $\mu < D$.

Substituting $\mu = \mu_{max}.s/(s + K_s)$ we obtain

$$s = \beta DKs/(\mu_{max} - \beta D) \qquad (25)$$

For the growth-limiting substrate balance we have

net rate of increase = input rate + feedback rate - output rate - substrate utilized for growth

that is, per unit of volume

$$ds/dt = Ds_r + aDs/(1-a) - Ds/(1-a) - \mu x/Y \qquad (26)$$

which reduces to the equation for ds/dt in the simple chemostat that is, $ds/dt = D(s_r - s) - \mu x/Y$.

Substituting $\mu = \beta D$ in that equation we obtain for the steady-state of x ;

$$s = (s_r - s)Y/\beta \qquad (27)$$

where $1/\beta$ is the concentration factor.

The critical dilution rate (D_c) will occur when $s = S_r$ and if $s_r >> K_s$, D_c, μ_{max}/β. The concentration of biomass (hx) in the dilute biomass effluent is obtained from the biomass balance;

$$F_s x = aF_s gx + cFgx + (1 - c)Fhx \qquad (28)$$

and substituting for F_s we obtain;

$$h = (\beta - cg)/(1 - c) \qquad (29)$$

The rate of output of biomass per unit volume of system will be

$$R = F_s x/V - aF_s gx/V \qquad (30)$$

and on substituting for F_s we find

$$R = \beta Dx \qquad (31)$$

The chemostat with feedback of biomass allow operations at dilution rates which greatly exceed the corresponding μ_{max}. The maximum dilution rate possible depending on the value of the feedback factor β.

By means of biomass feedback, the maximum output rate of biomass and product in a chemostat with a given medium can be increased. The system is advantageous also when the concentration of growth-limiting substrate has to be limited because of the formation of an inhibitory product.

1.3 - SUMMARY OF THERMAL AND ETHANOL TOLERANCE IN YEAST

1.3.1 - THERMAL TOLERANCE

Temperature exerts an important influence on all aspects of microbial physiology. Various aspects of thermal effects on yeasts may be found in earlier reviews on temperature effects on yeasts [18,19,20,21].

Microorganisms, unlike higher organisms, have no means of thermal regulation and consequently their intracellular temperature must be equal to that of the environment.

Elevated temperatures increase the rates of enzymic reactions However, individual species have a relatively narrow temperature range for growth of at most 40 to 50 degrees centigrades.

Therefore, all metabolic activities including specific growth rate, ethanol production rate, fermentation rate, nutrient requirement, substrate uptake, viability and other factors, are all influenced by temperature[22, 23, 24, 25,26,27].

The temperature range that will support microbial life is extensive, ranging from several degrees below the freezing point of water to a few degrees below its boiling point [28].

Microorganisms with a maximum temperature for growth (T_{max}) of greater than 50 ^{o}C are referred to as thermophilic, between approximately 20 to 50 ^{o}C are classified as mesophilic and organisms with a maximum temperature for growth equal or below 20 ^{o}C , are considered psycrophilic.

The most important species of microorganisms used in the fermentation processes belongs to the mesophilic group. Although there is a considerable biotechnological interest and advantage in employing thermophilic organisms.

The optimum temperature which support the highest maximum specific growth rate lies within the range values set by minimum temperature (T_{min}) and maximum temperature (T_{max})[29].

1.3.1.1 - EFFECT ON MEMBRANE COMPOSITION IN YEAST

Elevated temperatures are known to stimulate an adaptive response (homeosviscous adaptation) in bacteria by increasing the amount of unsaturated fatty acid, increasing the amount of branched fatty acid, decreasing the fatty acid chain lengths and the amount of cyclic fatty acid in the membrane in order to maintain constant membrane fluidity [30,31].

A similar adaptative response has been found with yeast strains from brewing in which a reduction in the fermentation temperature from 20 to 8 ^{o}C, produced an increase in fatty acid synthesis, particularly unsaturated fatty acid [32].

Unsaturated fatty acids, more so polyunsaturated, have lower melting points than saturated and so an increase in fatty acyl unsaturation would lead to enhanced membrane fluidity. This increases the resistance of a yeast cell to the entry of ethanol thereby enhancing tolerance to high extracellular ethanol levels.

However, this might allow the accumulation of intracellular ethanol, because ethanol efflux is also inhibited. Therefore, at higher temperatures the intracellular ethanol levels are more important for the inhibition of glycolysis and fermentation than for growth [33,34].

This implies that for enhanced ethanol production, the temperature of a batch process should be maintained relatively low. This is the case with sake brewing in Japan where a low temperature fermentation (about 8 degrees centigrade) allows the yeast to tolerate up to 23 % (v/v) ethanol.

However, increases in fermentation times at lower temperatures necessitate higher inoculum concentrations, as well as substantial increases in expenditure on cooling[19]. The ethanol yields of several species of yeast has been tested and shown to decrease with increasing fermentation temperature.

The mesophilic *Saccharomyces cerevisiae* strains tested (T_{max} for growth of about 42 ^{o}C) could produce up to 15 % (v/v) ethanol at 22 ^{o}C from glucose, but at 37 ^{o}C the maximum yield was 12 % (v/v).

In comparison, the most thermotolerant strain tested *Zygosaccharomyces fermentati*, produced no more than 7.5 % (v/v) at 37 ^{o}C.

The highest-yielding of the thermotolerant strains was *Candida brassicae* which produced an ethanol concentration of 5 % (v/v) at 42 ^{o}C [35].

Sa'-Correia and van Uden [36,37] found that the converse is also true in that the addition of ethanol to the cultures of yeast reduces their thermotolerance.

The addition of increasing concentration of ethanol to the yeast culture progressively depresses the T_{max} value for growth and increase the T_{min} value. Thus, ethanol reduces the temperature limits within which a strain of yeast is able to grow.

The addition of the maximum tolerable concentrations of ethanol 11 % (v/v) to a strain of *Saccharomyces cerevisiae* reduced the temperature range for growth by increasing the T_{min} value from 3 to a value of 13 ^{o}C, and decrease T_{max} value from 42 to 27 ^{o}C.

In *Kluyveromyces fragilis*, a thermotolerant yeast, the maximum tolerable concentration of ethanol, 8 % (v/v) was less than that for the mesophilic Saccharomyces cerevisiae strain and the effects of ethanol on the growth temperature were more severe.

In both the mesophilic and the thermotolerant strains, the addition of the maximum tolerable ethanol concentrations produced similar temperature range for growth. The thermotolerant yeast was, however, more sensitive to the effects of ethanol on the growth temperatures.

The effects of ethanol and temperature on membrane fluidity are antagonistic and therefore, increases in temperature enhance the inhibitory effect of ethanol and vice-versa.

Any adaptative response on the part of a yeast to elevated temperatures by reducing the proportions of unsaturated fatty acids present in the membrane would therefore increase the inhibitory effects of ethanol because unsaturated fatty acids (particularly polyunsaturated) when incorporated in the membrane have been shown to confer ethanol tolerance[38].

This might explain the differences in the ethanol tolerances of a mesophilic *Saccharomyces cerevisiae* strain and the thermotolerant *Kluyveromyces fragilis*.

Mesophilic yeasts have a greater proportion of polyunsaturated fatty acid in comparison with thermotolerant yeasts[39], and therefore more resistant to ethanol but less resistant to elevated temperature.

Leao and van Uden[40], measured the viability of a strain of *Saccharomyces cerevisiae* in the presence of a variety of alcohols, differing in hydrophobicity (chain length) and polarity (number of hydroxyl groups), at temperatures greater than the Temperature optimum for growth.

It was found that the greater the lipid solubility of the alcohol the more effective was the alcohol in enhancing thermal death. Therefore, the action of alcohols is non-specific and dependent upon their lipid solubility.

This is indicative of the membrane as being the principal target for the reduction in thermotolerance and the enhancement of thermal death in yeast by alcohols.

They also proposed that there are specific target sites (thermal death sites) present in yeast membrane which are temperature-sensitive. The number of the sites affected, and the degree of disorder caused by elevated temperatures on these target sites might result in thermal death.

Alcohols present in the membrane lipid bilayer at the vicinity of these target sites would increase the disorder in the region enabling a greater degree of disorder at lower temperatures resulting in reduction in T_{max}, and enhanced thermal death.

Ryu and Kwon[25], investigated the effects of fermentation temperature in the batch fermentation of high sucrose concentration 30 % (w/v) by a strain *Saccharomyces cerevisiae.*

The temperature optimum for growth was found to be 35 ^{o}C. However.the maximum final concentration of biomass were obtained only at 25 ^{o}C. The maximum ethanol productivity (g/L.h) was achieved near 40 ^{o}C, however, the maximum final ethanol concentration (140 g/L) and nearly complete utilization of sucrose ocurred between 20 and 25^{o}C.

Bottema et al.[41], showed that no phospholipid phase transitions ocurred over temperatures ranging from 15 to 40 ^{o}C in membranes from wildtype *Saccharomyces cerevisiae* because of the presence of ergosterol, the main sterol in yeast membrane.

In comparison, phase transitions ocurred between 22 and 25 ^{o}C in membrane from sterol auxotrophic mutants. Therefore, the presence of sterols seems to reduce the disordering effects of elevated temperatures on the fluidity membranes.

A larger number of temperature-sensitive cellular constituents or processes have been detected in a wide variety of yeast. Thermotolerant yeast might be expected to possess cellular constituents of greater resistance than those of mesophilic yeasts.

In mesophilic yeasts, for example *Saccharomyces cerevisiae* and *Candida albicans*, the specific growth and thermal death rates are associated at temperatures greater than Temperature optimum for growth (T_{opt}).

In this case, the reduction in specific growth rate at temperature in excess of T_{opt} is due to a combination of denaturation events, some of which slow down cell multiplication, and some that are lethal, resulting in cell death.

At the T_{max} value, cell multiplication ceases and at a higher temperature there is a progressive increase in thermal death rate. It is not known which cellular constituent or process, essentials for cell multiplications, is the most easily denaturated at the T_{max} value and is therefore the principal determining factor of the T_{max} value of a strain of yeast.

If the principal determinant of the T_{max} value in a thermotolerant strain were known, then this might enable the gene(s) involved to be characterised, cloned and used to transform a mesophilic strain to thermotolerant whilst still retaining the advantageous attributes of the mesophilic strain, such as ethanol tolerance and production [27].

The use of thermotolerant yeast is obviously of particular relevance in tropical, developing regions. A thermotolerant strain of *Candida pseudotropicalis* C-23 (revised nomenclature is *Kluyveromyces fragilis*; Lodder [42]), is used today in several Cuban distilleries where it performs well under tropical conditions in the industrial batch fermentation of molasses to ethanol.

The fermentative efficiency using C-23 strain was 10.2 % higher, which represented a decrease in residual molasses and increase in ethanol production, in comparison with the traditional Saccharomyces cerevisiae strains[43].

Industrial ethanol fermentation by yeasts are generally carried out in the range 25 - 35 ^{o}C since temperature close to 40 ^{o}C have adverse effects[18,20,44]. Although yeast growing at and above 40 ^{o}C have been reported [45,46], only limited reports of high-yield ethanolic fermentation by such organism are available [47,48,49,50],.

The potential economic benefits in ethanol production at higher temperatures (40 ^{o}C or above) have generated considerable interest in the selection of yeast strains resistant at elevated temperatures.

For example, it has been estimated that 30 - 35 % of the fermentation costs incurred in ethanol production at 32 ^{o}C are the result of the cooling process[51].

In distilleries in a tropical country such as Brazil, the energy costs of mechanical refrigeration are too high resulting in fermentation broth temperatures in excess of 40 ^{o}C. The use of thermotolerant strains in such situations could provide considerable economic advantages.

1.3.2 - ETHANOL TOLERANCE

Economically efficient industrial fermentations require high substrate concentrations for the production of high levels of ethanol prior to distillation. It is necessary however to select yeast strains which are osmotolerant, thermotolerant (to withstand increases in fermentation temperatures due to metabolically-generated heat) and ethanol tolerant[52].

It is well known that the ethanol produced during the fermentation of glucose by yeast eventually reaches a concentration at which it inhibits yeast metabolism,and the further conversion of substrate. This sensitivity of yeast to alcohol represents an important limitation in the production of ethanol by large-scale industrial fermentation[53]. The inhibitory effects of ethanol have been defined in a number of ways (see van Uden[20]).

Brown et al.[54], reported on the immediate effects of ethanol on yeast growth rate, fermentation rate and cell viability, whereas earlier researchers studied the effects of long term exposures to ethanol on yeast growth rate [55,56], and on cell viability of yeast cultures or suspension of cells [38] and thermal death [57].

Others examined the effects exposures to ethanol on glucose and ammonium transport system[58,59,60] and maltose transport [61].

The increase of intracellular ethanol levels in certain circunstances during a fermentation may be important for the inhibitory effect of ethanol on intracellular enzyme such as alcohol dehydrogenase[62].

From early examination of the effect of ethanol on the yeast glucose transport system it was noted that strains of Saccharomyces also showed unequal tolerances to high glucose concentration and that glucose tolerance varied directly with ethanol tolerance. Subsequent studies revealed that yeast cells of higher ethanol tolerance store less lipid and carbohydrate than yeast cells of lesser ethanol tolerance.

Yeast cells grown aerobically contain more lipid than anaerobically grown yeast cells and were shown to have less tolerance to ethanol than the latter[63].

The accumulation of ethanol in the microbial environment represents a form of environmental stress analogous to extremes of temperature and pH.

Maintaining the functional stability of the plasma membrane as an effective semi-permeable barrier controlling the exit and entry of various metabolites, and for the compartmentalization of essential processes within the cell is all important for the growth and survival of the cell in harsh conditions.

The simultaneous enhancement of ethanol and thermal tolerance in Saccharomyces cerevisiae has been shown to occur after a short preincubation time at high temperature[19].

Increases in temperature and ethanol concentrations have shown that both physical and chemical effects play a important roles a an inducers of transient synthesis of "heat -shock proteins" in yeast[64].

1.3.2.1 - EFFECT ON MEMBRANE LIPID COMPOSITION IN YEAST

Changes in membrane lipid composition are associated with changes in important membrane properties such as fluidity and permeability[65].

There is increasing evidence that the toxic effects of ethanol are,initially at least, expressed at the level of the plasma membrane and that the inhibition of sugar transport is the primary reason for reductions in yeast activity as ethanol concentration increases[66].

Therefore, since *Saccharomyces spp.* can tolerate relatively high concentrations of ethanol, these yeasts might be expected to possess different lipid compositions as well as different fatty acid content[67].

Thomas et al.[38], sucessfully altered the lipid composition in the plasma membrane of *Saccharomyces cerevisiae* by adding combinations of sterols and fatty acids to the nutrient medium containing anaerobically grown cells. In all cases it was found that linoleate (polyunsaturated fatty acid) with sterol, when incorporated in the plasma membrane, conferred greater tolerance to ethanol than oleate (monounsaturated fatty acid).

Those cells enriched with ergosterol or stigmasterol which have unsaturated bonds at C_{17} in the side chain, and linoleate showed greater resistance to ethanol than those enriched in campesterol or cholesterol, which have saturated side chains and linoleate. Ergosterol with palmitoleate ($C_{16:1}$) was better at conferring ethanol tolerance than with either oleate ($C_{18:1}$) or cetoleate ($C_{20:1}$).

Increased fermentation rates and enhanced ethanol tolerance has been achieved using similar techniques with *Pachysolen tannophilus*, a yeast which ferments the pentose sugar xylose to ethanol, after the addition of exogenous ergosterol, linoleate and Tween-80 to the nutrient medium of semi-aerobically growing cells.

Analysis of fatty acid composition before and after lipid supplementation revealed that *Pachysolen tannophilus* had in fact incorporated linoleate and ergosterol into the plasma membrane[68].

Sterols present in membranes may perhaps offer some steric hindrance to the movement of molecules within the membrane so maintaining some degree of order[69].

The presence of unsaturated fatty acids in the membrane, however,enhance fluidity. Therefore, the fluidity of a membrane is determined by the ratio of unsaturated fatty acid to sterol.

In industrial fermentations substrates such as molasses or grain preparations may contain lipids, unsaturated fatty acids, and sterols, consequently aeration may not be required. However, development in the use of more purified media may lead to reductions in these essential nutrients which therefore necessitates a certain amount of aeration [70].

The commercial yeast strains presently used in industrial ethanol production and in the brewing industry display relatively high ethanol tolerance as a result of a genetic selection brought about by prolonged exposures to ethanol.

Brown & Oliver[71], have developed an ingenuous feedback system in which the addition of ethanol to a continuous culture of *Saccharomyces uvarum* is used to control growth rate. The growth rate is monitored by the output of CO_2 which controls the rate of inflow of ethanol, maintaining a constant selection pressure for ethanol tolerance.

They obtained mutants with an ethanol tolerance up to a level of 12 % (w/v). Jones & Greenfield[72], acclimatized *Saccharomyces cerevisiae* to 7 % (w/v) ethanol by growth in a chemostat at 30 ^{o}C, with an inlet of 20 % (w/v) glucose and a residence time of 30 hours, for 16 residence times.

The viability of these cells on incubation with various concentrations of ethanol at 25 ^{o}C for up to 75 hours was increased by a factor of 40 compared to non-adapted cells. However, adapted cells demonstrated increased lag phase when grown on complete medium without ethanol in comparison to non-adapted cells.

These results imply that previous culture history has a significant effect on the response of cells to ethanol, but the genetic basis of such tolerance remain unknown.

Walker-Caprioglio et al.[73] found that lipid supplementation of the nutrient medium of sterol auxotrophic mutants did not reduce the inhibitory effects of ethanol, and the lag phase did not enable the selection of more ethanol resistant forms but did enable the yeast to overcome the ethanol induced inhibition of growth.

Reductions in the duration of lag phase was observed however, for the cells grown in ethanol-containing glucose medium which had been conditioned by pre-growth with yeast.

This suggests that yeast cells excrete one or more components into the medium which confer resistance to the ethanol -induced inhibition of growth.

Ethanol -induced reductions in membrane fluidity inhibit the activities of membrane-associated proteins.

However, any increase in the unsaturation of the fatty acyl residues in the phospholipids surrounding membrane proteins may reverse the effect of ethanol, by enhancing the fluidity of the lipid bilayer[74].

1.3.2.2 - THE EFFECT OF OSMOTIC PRESSURE IN YEAST

The utilization of a high gravity wort fermentation are an important consideration for the production of high ethanol concentrations prior to distillation[75].

The metabolic activity of yeasts increases with increasing substrate concentration until an optimum is reached. The optimum substrate concentration is strain dependent. Above the optimum there is a marked reduction in the yeast activity followed by plasmolysis at higher concentration (approximately 14 % (w/v) due to osmotic effects[76]. Panchal & Stewart[77], observed the effects of increasing media osmotic pressure by addition of sorbitol, a non-metabolizable sugar, to the sucrose wort of a semi-aerobically growing brewing strain of *Saccharomyces uvarum*.

Increasing the osmotic pressure above the optimum resulted in a increase in the level of intracellular ethanol, a decrease in ethanol excretion, and an increase in the excretion of glycerol, a by-product of fermentation. This resulted in a net reduction in ethanol production and reduce growth and fermentation rates.

Therefore, reduction in the activity of yeasts in high gravity worts might be due to inhibition by elevated intracellular ethanol concentration and the consequent re-direction of carbon into glycerol, rather than ethanol production.

Extracellular ethanol or the depletion of essential nutrients does not appear to be the reasons for yeast inactivity under this conditions. Increasing the osmotic pressure by addition of salts to the nutrient medium of *Saccharomyces cerevisiae* also results in increased glycerol production from dihydroxyacetone phosphate and excretion at the expense of ethanol.

However, increasing the concentration of salts in the medium tended to produce increase in ethanol productivity and a decrease in cell viability.

This trend is explained by postulating that, as the osmotic pressure of the medium increases, the maintenance requirements of the cell culture increases.

Glycerol probably then functions as a non-toxic osmoregulator to combat the elevated leakage of ions into the cell[78].

Ethanol measurements during a fermentation with *Saccharomyces cerevisiae* revealed that initially the internal ethanol concentration was the same as the external but that at the end of fermentation internal ethanol levels fell considerably[79] .

Therefore, the passage of ethanol from inside the cell to outside must be against a concentration gradient, contrary to the opinion derived from earlier estimations of intracellular ethanol by Thomas & Rose[80].

This led Desari and co-workers[81], to propose that might be an active transport mechanism for ethanol efflux. If this were true, the inhibitory effects of ethanol on protein-mediated transport across the plasma membrane would reduce the efflux of ethanol and lead to the accumulation of intracellular ethanol.

However, subsequent studies, resulting in further reductions in intracellular ethanol estimations showed that there was, in fact, no difference between internal and external ethanol concentration during the fermentation[81].

Therefore, ethanol diffuses freely across the membrane and rapidly reaches equilibrium with the outside. It seems unlikely that accumulation of internal ethanol is specifically important in the inhibition of fermentation and growth in yeasts (for reviews see van Uden [15]).

Ethanol is an amphipathic molecule; containing both a hydrophobic and a hydrophilic region,and as such may penetrate the hydrophobic core of the membrane lipid

bilayer where, because ethanol is relatively polar,there is a reduction in hydrophobicity at that particular region in the core which enables the solubilization of the other polar molecules. This effect spreads throughout the membrane resulting in decreased fluidity and increased permeability.

Ethanol-induced dehydration and denaturation of membrane proteins and of proteins on the interior of the cell, after the passage of ethanol through the membrane, further reduces the functional integrity of the plasma membrane[82]. The fermentation of sugar to ethanol in yeast is usually considered to be an anaerobic process, but in aerobic conditions with high sugar concentrations, growth in *S. cerevisiae* proceeds as a result of the a "anaerobic" fermentative pathway due to catabolite repression and inactivation of enzymes in the TCA cycle and oxidative phosphorylation[83].

Inactivation of respiration leads to a reduction in the levels of ATP and citrate which activate glycolysis and fermentation (Crabtree effect, that is, repression of respiratory activity by glucose under aerobic conditions and subsequent deregulation of glycolysis with formation of ethanol). However, in limiting sugar concentration and excess of oxygen the catabolite repression and inactivation is alleviated and the TCA cycle and oxidative phosphorylation are activated. Respiration then leads to an increase in the levels of ATP and citrate which

inhibits glycolysis and fermentation (Pasteur effect, that is, inhibition of the glycolytic pathway in the presence of oxygen; manifested as inhibition of alcoholic fermentation). However, the Pasteur effect is also prevalent even in anaerobiosis when nitrogen levels in the growth medium become depleted[70].

In fact, the Pasteur effect may be considered as a secondary facet of yeast metabolism as it only occurs under special experimental conditions, for example in a chemostat culture when growth is limited by very low concentration of sugar, or in stationary phase (resting cells) when sugar is catabolised in the absence of a nitrogen source. Barford & Hall[84], observed that the repression of respiration during batch growth was a consequence of a very slow adaptation to fully respiratory conditions. In carbon-limited continuous culture, where sufficient time was available for this adaptation, no repression of respiration was observed with either glucose or galactose as the carbon source.

These results nevertheless do not eliminate the possibility that repression is caused by sugar concentration higher than those obtained in carbon-limited continuous culture, although comparison of galactose and glucose batch and continuous culture experiments would suggest that this was unlikely.

However, much work will be necessary to arrive at a proper interpretation of the many data in earlier publications.

Too many metabolic steps are normally involved in regulation of growth under excess of carbon (batch) or carbon limitation (chemostat). Also, strain dependence, as well as carbon source specificity under aerobic and anaerobic conditions, are responsible for the confusing scene of this regulatory phenomenon.

According to Ryu et al.[85], the most important areas of research in ethanol fermentation have been:

(i) Increased ethanol concentration and specific ethanol production rate.

(ii) Improvement of ethanol tolerance of yeast.

(iii) Development of a continuous ethanol fermentation process using high density cell culture.

One of the fundamental difficulties in assessing ethanol tolerance in various yeasts is that there is no universally accepted technique of measurement or definition of ethanol tolerance. Ethanol has three major effects on yeast cells. It inhibits cell growth, cell viability and fermentation, although to different extents. Thus, the definition of ethanol tolerance dependes on which parameter one is reffering to.

The possible target sites of ethanol in yeast cells are depicted in Fig. 1.3.2. One of the major target sites of ethanol in yeast and other microorganisms is the plasma membrane, as well as the membrane of the various cellular organelles.

Despite centuries of experience, the processes of ethanol production, excretion and their regulation are still not entirely clear (for reviews see D'Amore et al.[86,87]).

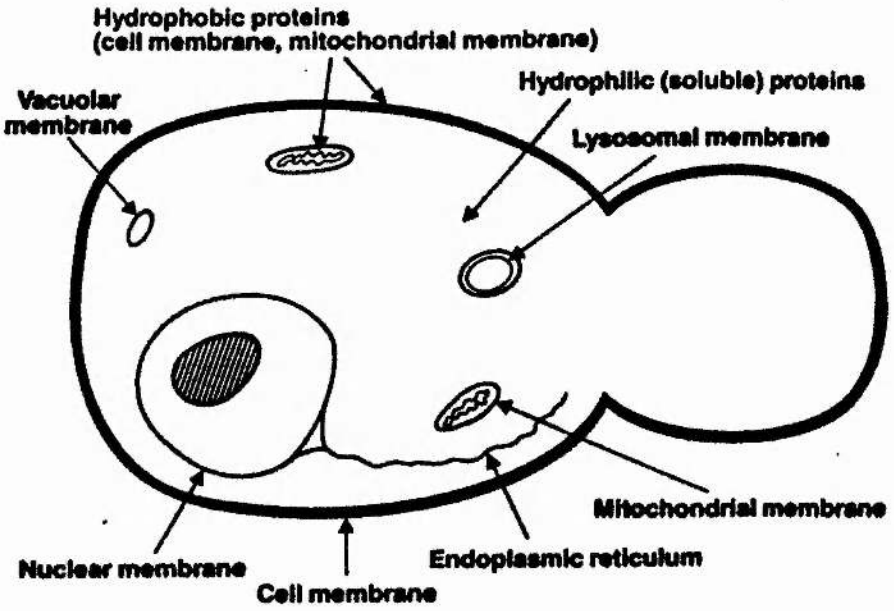


Fig. 1.3.2. - Possible target sites of ethanol in yeast cells. (D'Amore,1987).

1.4 - FERMENTATION PROCEDURE

Fermentation procedures for ethanol production can be divided into two broad categories:

(1) Batch process.

(2) Continuous process.

1.4.1 - BATCH PROCESS

Most microbial processes for ethanol production involve batch growth. In a batch system, a vessel is filled with starting materials including the substrate (usually liquid medium) of appropriate composition and an inoculum of living cells. Nothing further is added to the culture or removed from it as growth proceeds. The fermentation takes place over a period (depending on the strains used, the size and activity of inoculum) that can range from 4 hours upwards. When the fermentation is completed, the amount of ethanol makes up from 8 to 12 % (w/v) of the spent medium, which also includes by products, unconsumed nutrients and cells. Ultimately the fermenter is emptied, probably only cleaned, refilled and then a new batch is started.

Batch systems are simpler than continuous ones but suffer from the usual disadvantages of slow fermentation in the early stages before the yeast population has built up. These process can account for up to 20 % of cycle time. However, that fact has been overcomed in many industrial fermentation processes by the use of a large inoculum.

The batch fermentation may be used to produce biomass, primary and secondary metabolites.

For biomass production, cultural conditions supporting the maximum cell population would be used; For primary metabolite production conditions to extend the exponential phase accompanied by product excretion and for secondary metabolite production, conditions giving a short exponential phase and an extended stationary, or production phase, or giving a decreased growth rate in the log phase resulting in earlier secondary metabolite formation.

This type of process has permitted industrial development without recourse to detailed studies of complex biochemical reactions involving poorly understood reaction kinetics and variables, such as metabolic control, and substrate and product concentration dependence. A consequence of this approach is that many commercial processes are overdesigned in the sense that they are carried out under suboptimal conditions [88].

1.4.2 - CONTINUOUS PROCESS

Industrial fermentation by continuous culture technique has several advantages over batch fermentation processes for instance, reduced reactor volumes and reduced down-time, (harvesting, sterilising, recharging) greater uniformity, ease to control, all resulting in significantly higher productivities.

In a continuous process the nutrients are continuously added to the fermenter vessel and the product of the reaction are continuously removed. Because continuous reactors lose cells in the effluent stream their productivity is limited by the growth characteristics of the organism and depends on the dilution rate employed.

One way to overcome the above limitation is recycling the biomass (feedback), that is, concentrating the biomass from the effluent and then returning a fraction to the fermenter. The maximum possible dilution rate in a system with feedback of biomass is greater than in a conventional continuous system. Another limiting factor is the inhibition effect of product (ethanol) on cell growth and fermentation rate (see section 1.3.2.).

A number of different techniques have been investigated to overcome the above limitation.

A cell recycle technique associated with vacuum fermentation was developed for continuous ethanol production by Cysewski and Wilke [89] to achieve high cell densities and rapid ethanol fermentation rates. Through that system ethanol productivity increased as much as twelvefold over conventional continuous fermentation.

However, a high energy input is required to operate this system,as well as the vacuum process require a higher capital costs, more sophisticated control devices and a higher operator skills. Much more emphasis has been placed on this consideration in the U.S. than in Brazil where a low technology approach has been adopted.

In terms of Northeast Brazilian ethanol production it seems unrealistic as a possible technology. Continuous systems offers the advantage of improved volumetric efficiency due to operating at a constant rapid fermentation rate and thus lower capital costs.Yeast costs are greatly reduced since inoculation has to be performed only once for each extended fermentation cycle.

Control over the fermentation is improved due to steady state operation leading to a consistent product and improved operating efficiency distillation. Peak loading an ancillary equipment is eliminated allowing reduced capacity.

It is claim that single vessel continuous systems are more resistent to bacterial contamination than batch or cascade systems because they operated at high ethanol concentration.

Most continuous systems in operation are of the multi-vessel type, usually employing four to seven vessels.The first vessel is aerated to promote the yeast growth the other have carbon dioxide mixing.

Anti-foam is added to the first vessel. Cooling is by external heat exchanger when the flow from the tank is pumped, or by external coils on the tank where transfer is by gravity. The substrate is fermented progressively as it passes from tank to tank, the first tank having an ethanol concentration of about 4 % . Yeast growth can thus take place at a relatively low ethanol concentration and in the last vessel, where ethanol concentration may be high enough to inhibit growth, conversion of the remaining sugar can occur without growth. By maintaining a very high concentration in the system, growth is minimised and ethanol yield enhanced [89].

The development of a new and improved bioprocesses depends on the realities of the commercial world: There must be a profit in the production of a particular product; The process must have a significant advantage over the existing or potential chemical process; and the technical difficulties involved in bringing the new process to commercial production must be solvable within the framework of existing or newly emerging technology.

One other reality, which is, perhaps, the most stringent is the willingness of the chemical (and other industries) to replace a traditional, operating, capital - equipment - in - place technology with one that requires new substantial expenditure [90].

However, the application of the new technology to energy industries in Brazil depends on its own social needs and conditions in science and technology.

Unfortunately, the Brazilian government did not maintain its efforts on scientific and technological development for many reasons;

Firstly, due to a lack of cooperation and coordination from the governmental authorities in maintaining their projects and incentives for research and development. Secondly, to the low income received by University teachers, researchers and technicians. Thirdly, it seems to be a serious matter, that is, nowadays few Brazilians trust or believe in government plans, decrees or projects.

For these reasons, most of Brazilian industries will remain at least 10 years behind with regards to technological progress compared with some developed countries such as Japan, USA, France and some other countries in Europe.

1.4.3 - CURRENT PRACTICE IN NORTHEAST BRAZILIAN SUGAR FERMENTATION UNITS.

Ledingham & Kinghorn [91] and Ledingham [92], reported on an extensive survey of fermentation units attached to sugarmills in the Northeast Brazilian State of Alagoas, see Figure 1.4.3 (a),(b),(c) and (d).

As these report are not widely available the main findings are reported here. Some 90 % of the fermentation plants are run on a batch operation process using a 60,000 L yeast cream inoculum to a final volume of 200,000 L. The most successful plants operating show 4 - 6 hours fermentation periods in which a cane juice initial (Brix = 12^0) was fermented out.

Substrates also included cane molasses (as locally available, as a supplement to cane juice. Such supplements frequently led to foaming problems or yeast clumping problems with consequent yield reductions. Additional, plainly, evident technical problems included :

a) Poor hygiene in the cane washing/milling operations leading to high Leuconostoc/Lactobacillus infections and consequent diversion by fermentation of sugar from alcohol to organic acids.

b) Lack of effective fermenter cooling (due to imported energy costs/ bagasse energy insufficiency) leading to high temperatures (above 40 ^{0}C) with consequent substantial evaporative loss of product in the open-topped fermentation vessels. Additionally such temperatures adversely affect yeast viability.

c) Lack of effective management of the fermentation plant. Managers range from the unqualified to the uninterested and in general, had no interest in fermentation improvements. Reliable data on fermentation performance was almost non-existence.

d) Frequently process disruptions to what is essentially a continuous process (8 x 200,000 L fermenters as a rotating schedule) led to bottlenecking and queing which also affected productivity.

While some of the problems (e.g. Lack of cooling) associated with alcohol-fermentations in Northeast of Brasil are those to be expected in an energy-restricted rural operation with climatically-imposed high ambient temperatures (25 - 35^{0}C), others (e.g. poor hygiene, use of inappropriate strains and lack of effective management) are more addressable.

There is an urgent need remaining for yeast strains which tolerate relatively high temperatures (above $40^{0}C$) and still show high alcohol productivity.

An increasing number of continuous systems (with 4 x 200,000 L fermenters operating in cascade) are being brought into operation.

At the present time, however,(05/95) political and economic considerations (crude oil prices) make the future of the Brazilian Proalcool program uncertain.

Urgent attention to fermentation yield improvements could help to reduce that uncertainty.

Fig.a

Fig. b

Figure 1.4.3 a,b - Fermentation Units from Northeast Alcohol production plants. Substrate arriving at and being transported to the fermentation units.

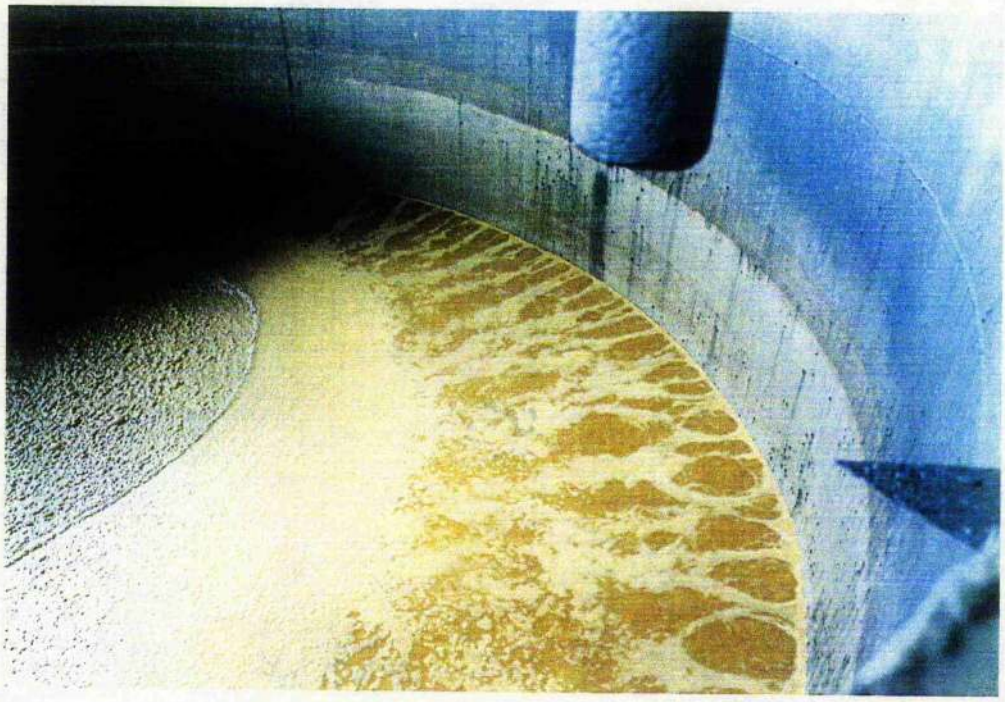


Fig. c

Fig.d

Figure 1.4.3. c, d - Shows Batch fermentation process (c) (open vat 200.000 L capacity) and the unit of distillation (d).

1.5 - AIMS

The Aims of this project fall into 2 groups:

1.5.1 GENERAL OBJECTIVES

A general objective was to acquire practical skills in a variety of fermentation techniques from batch to chemostat with feedback, including skills in apparatus construction (framework, glassware, instrumentation and basic eletronics for control).

1.5.2 SPECIFIC OBJECTIVES

To study the physiology of selected ethanol producer strains from Northeast of Brazilian fermentation plants and particularly through longterm chemostatic culture with feedback, to investigate their ethanol productivity and thermal tolerance and hence, their suitability as process organisms in these plants.

2. - MATERIALS AND METHODS

2.1 - MICROORGANISMS

Three strains of yeasts were used in this project, *Saccharomyces cerevisiae* (42- Fleischmann and PLA 851) and ***Saccharomyces boulardii*** (IZ 1904).

These strains are currently used alcohol producer strains employed in production plant in Alagoas State (NE - Brazil). The strains were a donation from CRPAA/NATT to Dr.W.M. Ledingham (Department of Biochemistry and Microbiology, University of St.Andrews - Scotland).

2.2 - MEDIA

2.2.1 - INOCULUM MEDIUM

Sucrose	2.0 g
Yeast extract	0.5 g
KH_2PO_4	0.5 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4.7H_2O$	0.1 g
Final volume	100 ml

2.2.2 - BATCH MEDIUM

Sucrose	12.0 g
Yeast extract	3.0 g
KH_2PO_4	3.0 g
$(NH_4)_2SO_4$	3.0 g
$MgSO_4.7H_2O$	0.6 g
Final volume	600 ml

2.2.3 - CONTINUOUS CULTURE MEDIUM

Sucrose	400 g
Yeast extract	100 g
KH_2PO_4	100 g
$(NH_4)_2SO_4$	100 g
$MgSO_4.7H_2O$	20 g
Final volume	20 Litres

Batch and continuous experiments were carried out using media containing different concentrations of sucrose as described in section 2.6 .

2.2.4 - MAINTENANCE MEDIUM

Strains were maintained in media of the following composition:

Sucrose	2.0 g
Yeast extract	0.5 g
KH_2PO_4	0.5 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4.7H_2O$	0.1 g
Agar	2.0 g
Final volume	100 mL

Following growth for 48 h at 30 ^{o}C, the organisms were maintained in culture plates at 4 ^{o}C and subcultured every two months . Periodically a sample from the culture was checked for contamination and purity by Gram staining and microscopic examination.

2.2.5 - STERILISATION OF GROWTH MEDIA

Media for batch and continuous culture were sterilised in two parts : A solution of sucrose and $MgSO_4$ was autoclaved at 5 p.s.i. for 20 min. and the remaining components were autoclaved at 15 p.s.i. for 25 min. This procedure served two purposes : First, it prevents caramelisation of the sugar. Second, it prevents the following reaction :

$$MgSO_4 + KH_2PO_4 \longrightarrow KH_2SO_4 + MgPO_4$$

Which would have resulted in the precipitation of insoluble $MgPO_4$. The ocurrence of either of these would have changed the composition of the medium as well interfering with the absorbance measurements.

2.3 - APPARATUS COMPONENTS

An apparatus for continuous culture fermentation with cell recycle was constructed on an angle iron framework. The apparatus consisted of a medium reservoir, fermenter flask, sedimentation vessel and product receiver or disposal vessel.

The culture vessel had a head plate with multiple ports through which growth medium was fed at a constant flow rate. Other ports held a thermostatted heater and a sample collecter.Two peristaltic pumps and a magnetic stirrer were fitted providing a constant flow rate, efficient mixing and uniform distribution throughout the fermenter vessel. Oxygen was not supplied neither was the pH controlled. It was, however, monitored at least at 12 hour intervals.

The main function of a fermenter is to provide a controlled environment for the growth / product formation of a microorganism. In constructing a system for continuous culture, with and without recycle of biomass, a number of points must be considered:

(I) The vessel should be capable of being operated aseptically for long periods and remain reliable in long term operation.

(II) An adequate system of temperature control must be provided.

(III) Adequate aeration and agitation should be provided to meet the metabolic requirements of the microorganisms.

(IV) Sampling facilities should be provided.

(V) The cheapest materials and systems compatible with satisfactory results should be used.

We built up this apparatus based on the idea that it should be readily re-constructable in third world laboratories with limited financial and technical resources.

2.4 - EQUIPMENT

a) - A borosilicate glass vessel of 1 litre capacity working volume of (690 ml) with a round top plate modified and adapted to support eight entry holes, where the heater, temperature sensor, inlet of medium, inlet of recycled biomass, thermometer, condenser and and outlet of gases are fitted into it.

The outlet from the fermenter is a borosilicate tube, which was fixed to the middle of the fermenter wall giving some advantages in terms of saving one peristaltic pump (see figures 2.4 a, and b).

b) - Two peristaltic pumps (Edmund Buhler, model type MP1 and MP2, supplied by Northern Media Supply LTD. England) with a flow rate capacity ranging from 10 to 600 mL/h.

c) - A magnetic stirrer powered by a Citenco motor drive model type KQPS/22, with a speed ranging from 50 to 300 rpm.

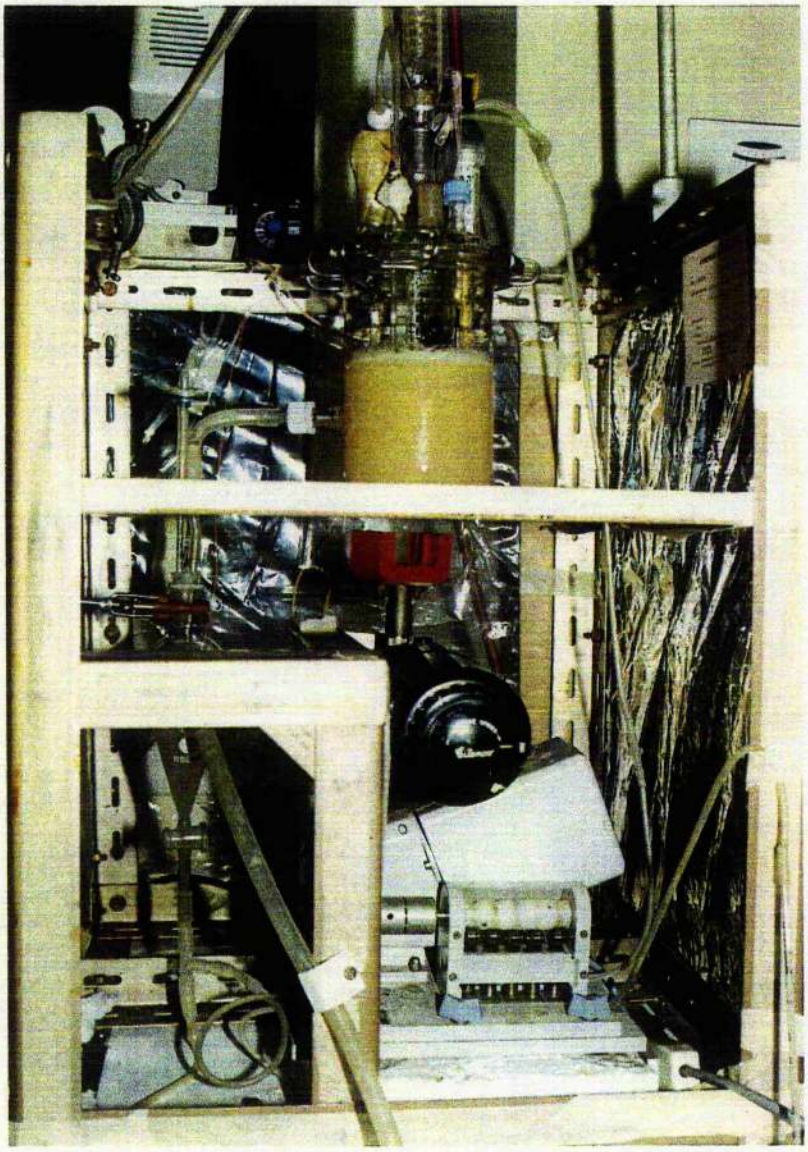


FIGURE 2.4 a - Shows the equipments used in the continuous culture with feedback of biomass experiments.

d) - The sedimentation vessel (Cell concentrator), is a cylindrical - conical vessel, that is, a decanter vessel modified, type Bs 2021 with 100 ml capacity (PYREX).

e) - Temperature controller model RS, Stock n^{o}. 344.625 consists of a Relay contact (output), and a platinum resistence thermometer (sensor) ranging from 0 to 100 ^{o}C. Supplied RS COMPONENTS LTD.

f) - A heater (ceramic covered resistor of 11 Ohms) operating at 14 W supplied by RS power 12 Volts step down from 240 VAC by a voltage transformer. Supplied by RS COMPONENTS LTD - Corby, Northants England.

g) - Spectrophotometer UNICAM - model.SP 600 Series 2. Made Unicam Instruments, Cambridge - England.

h) - Centrifuge Eppendorf -(5413), Geratebau - Netheler + Hinz GmbH - U/min (r.p.m. 11.500). Made in West Germany.

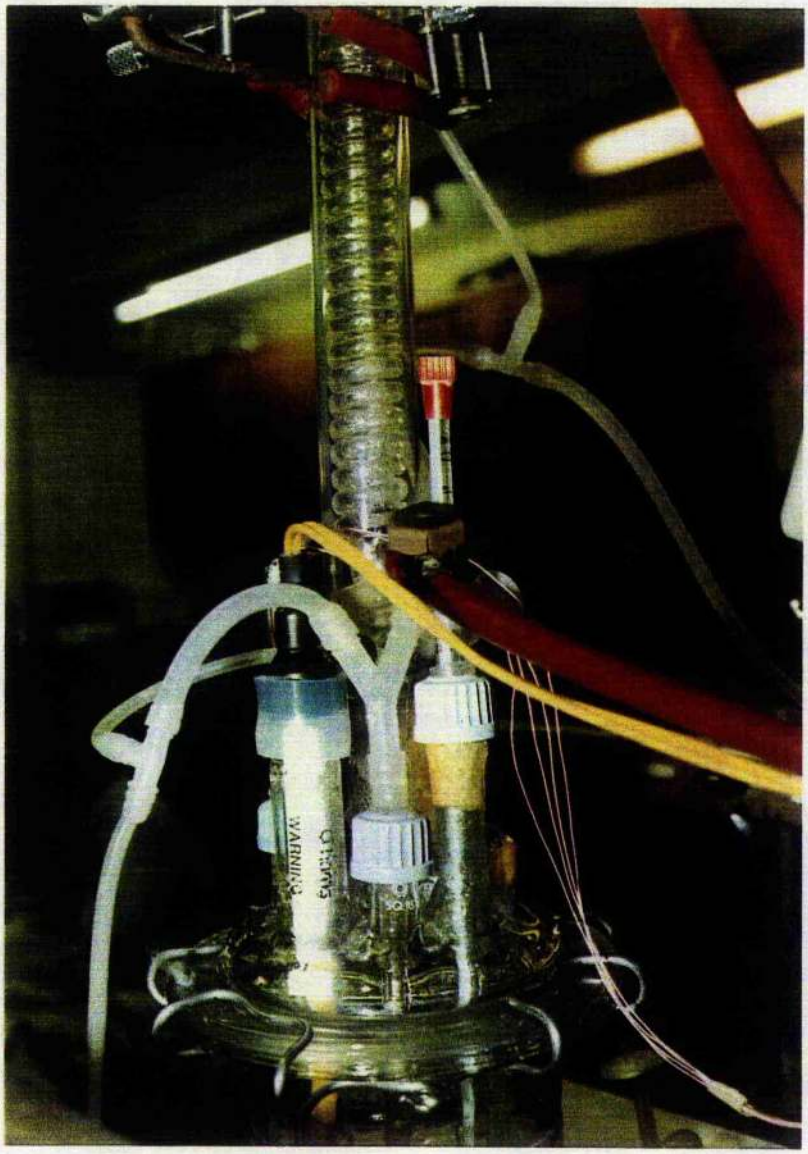


FIGURE 2.4 b - Shows the round top plate of the fermenter vessel modified and adapted for the continuous culture experiments.

i) - Gas-liquid Chromatograph - model PYE Series 104, supplied by Unicam Instruments. Cambridge - England.

j) - Microscope model Kyowa - medilux - 12 made in Japan. Supplied by Finlay Microvision. Warwickshire - UK.

2.5 - CHEMICALS

Ethanol, Isopropanol and 3,5 - dinitrosalysilic acid were provided by BDH CHEMICALS LTD - POOLE, ENGLAND. All chemicals used were of Analar grade.

2.6 - FERMENTATION EXPERIMENTS

2.6.1 - GROWTH CONDITIONS

Yeast cells were pregrown in a 250 mL conical flask in 100mL of sterile fermentation medium closed with a cotton plug for 18 hours in a rotary shaker. The temperature was controlled at 30, 35 or 40 ^{o}C for each different set of experiments. The medium containing the same components as stated in 2.2.2.

2.6.2 - BATCH EXPERIMENTS

The batch experiments were carried out in a fermenter glass vessel (QUICKFIT, 1 Liter capacity) with 630 mL of sucrose based medium. The inoculum was 60 mL in late log phase on either 2, 5, 10 and 20 % (w/v) sucrose. This gave a final working volume of 690 mL. A mechanical antifoam breaker device was built in it to prevent interference of foam with the flow rates in the continuous experiments. A motor driven mechanism for rotating the magnetic stirrer with variable rotation speed was fitted in order to maintain a homogeneous culture and micro-aeration in an environment without external oxygen supply.

The filters fitted at the top plate were used to make easier the inlet and outlet of the gases. Each strain was batch cultured as described above, and the growth rate were measured by determining the biomass concentration every hour through out the period of exponential growth. This was carried out by measuring the absorbance of culture sample at 610 nm in a UNICAM - SP 600 spectro photometer.

The relationship between biomass concentration and absorbance up to certain limits is linear, so that the absorbance can be converted to biomass concentration by reference to a calibration plot.

2.6.3 - CONTINUOUS EXPERIMENTS

The continuous experiments were carried out in the same glass fermenter vessel as stated in section 2.6.2. Continuous culture experiments with feedback of biomass were carried out using a external glass vessel to concentrate the biomass by gravity (see section 2.4, d). The feed solution was pumped to the reactor and allowed to stand for 1 hour before the test flow rate started and the medium left the fermenter vessel by an overflow pipe inserted through a side arm. The temperature was maintained at 30, 35 or 40 ^{o}C.

The continuous stirred tank fermenter (CSTF) with feedback of biomass operated at various flow rates from 0.05 to 0.35 h^{-1}. At each dilution rate the effluent was sampled at 12 hour intervals and analysed for sucrose, ethanol and biomass concentrations by assaying three successive samples.

All operation were done under aseptic conditions. Usually,a chemostat experiment is carried out after previous microbial growth in batch system. When the culture achieves the exponential phase the flow of the feed medium is connected. Under steady state conditions the mean generation time (t_d = doubling time) is calculated by the following equation:

$$t_d = (\ln 2/\mu) \qquad \text{where } \mu = D$$

The flow rate was determined measuring the time (min) to fill a 10 mL volumetric vessel. The dilution rates were calculated dividing the flow rate by the volume of culture inside the fermenter (690 mL) in the continuous experiments without feedback of biomass by the following expression; D = F/V.

However,for the continuous experiments with feedback of biomass, the recycled stream (R), was incorporated into the above expression. Then it becomes D'= F + R/V.

Where D' is the overall dilution rate, F is the flow rate of feed medium, R is the flow rate of recycled stream, and V is the volume of the fermenter.

2.7 - SPECIFIC GROWTH RATE

The maximum specific growth rate of each strains was determined using the following approaches:

2.7.1 - SPECIFIC GROWTH RATE DETERMINED THROUGH A BATCH EXPERIMENT.

The specific growth rate in a batch culture was calculated using the following equation:

$$\ln x = \ln x_o + \mu t$$

where x_o is the biomass when $t = 0$

The plot of ln x against time (t) in exponential growth will be a straight line in which the slope is equal to μ.

2.8 - ANALYTICAL METHODS

2.8.1 - DETERMINATION OF BIOMASS CONCENTRATION

In order to determine biomass concentration from absorbance it was necessary to prepare a calibration plot as follows: One of the strains was cultivated in 630 mL of sucrose based medium for 24 hours. The cells were harvested by centrifugation on MSE - HS18 centrifuge at 1000 x g for 10 minutes at 4 ^{o}C.

The supernatant was discarded and the cells were resuspended in 50 mL of distilled water and then recentrifuged and the process was repeated three times. Finally, the cells were resuspended in 10 mL of distilled water to give a thick but pipettable solution.

Three 1.0 mL aliquots of the suspension were transferred to three vials accurately weighed and placed to dry in an oven at 120 ^{o}C overnight and the samples were left to cool in a desiccator and reweighed and the mean dry weight was determined.

2.8.2 - PREPARATION OF A BIOMASS CALIBRATION PLOT

A further 1.0 mL aliquot of cell suspension was taken and a serial dilutions made such that the absorbance at 610 nm covered the entire range from 0 to 0.6 . The absorbance of the serial dilutions was read against a blank of distilled water. Since the accurate dry weight in a 1.0 mL of cell suspension was known, the equivalent dry weight of each dilution could be calculated.

A plot of dry weight of the dilutions against their absorbances produces a linear relationship between the turbidity measurement and the organism dry weight. The whole procedure was done twice and the average taken (see fig. 2.8.2).

2.8.3 - BIOMASS MEASUREMENT DURING EXPERIMENTS

The absorbance at 610 nm of the samples removed from the culture were measured. For readings greater than 0.6 a 1/10 dilution was used since the relationship biomass concentration versus absorbance is linear for abosorbance less than 0.7 . The absorbance readings were then converted to biomass concentration by reference to the calibration plot.

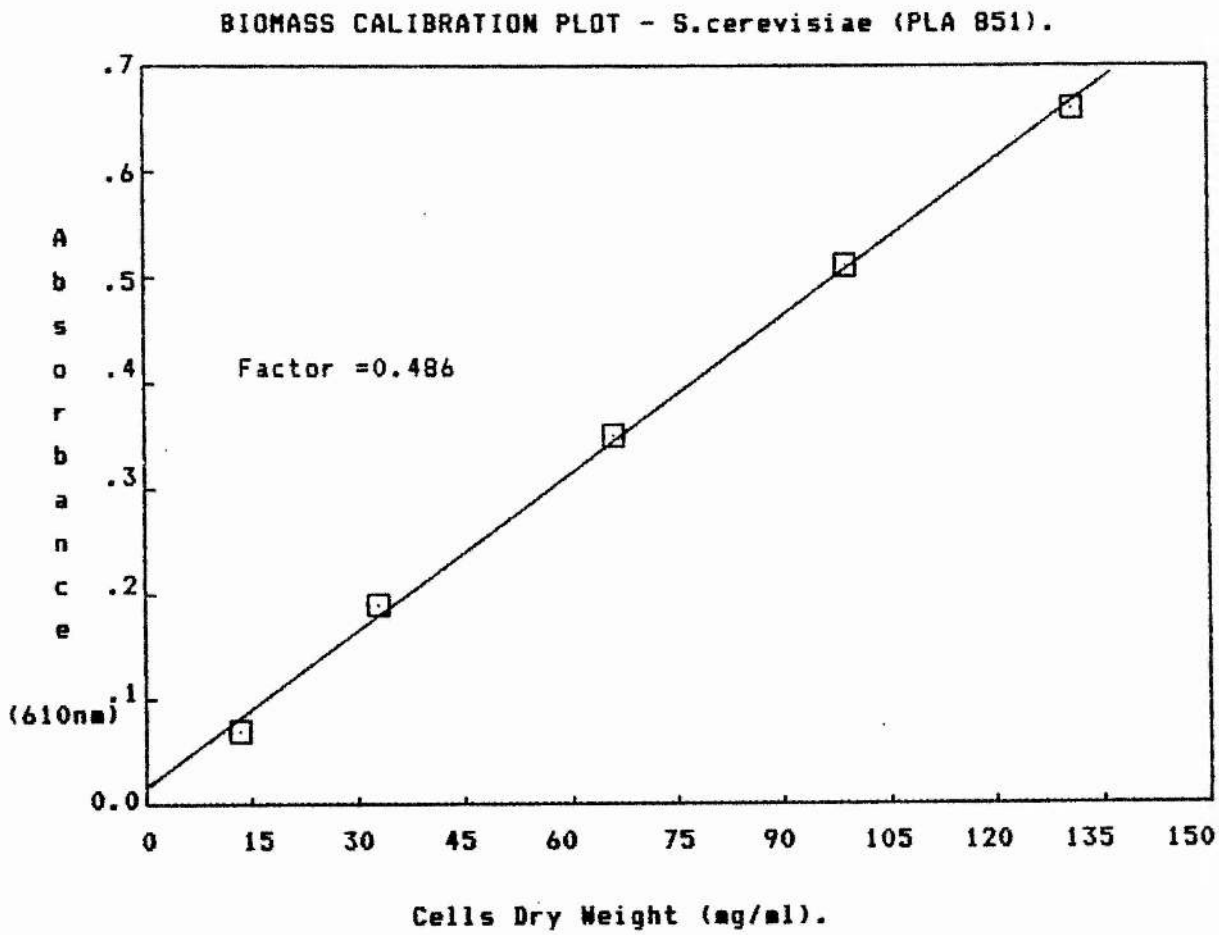


FIGURE 2.8.2. - CALIBRATION PLOT OF THE CULTURE TURBIDITY AGAINST BIOMASS DRY WEIGHT

2.9 - DETERMINATION OF SUCROSE CONCENTRATION

2.9.1 - PREPARATION OF DNSA REAGENT

5.0 g of reagent grade 3,5 - dinitrosalycilic acid was dissolved in 100 mL of 2 M sodium hydroxid solution at 70 ^{o}C. Then 150 g of potassium sodium tartrate was dissolved in 100 mL of distilled water at 70 ^{o}C. After that the solutions were mised and the final volume made up to 500 mL with distilled water and stored in a dark glass bottle.

2.9.2 - PREPARATION OF SUCROSE CALIBRATION PLOT

A standard set of sucrose solutions ranging from 0 to 20 mg/mL were prepared . A series of 10 tubes containing 0.5 mL of sucrose with different concentrations were done and then 1.0 mL of 0.75 M hydrochloric acid was added into it and boiled for 30 min.

After that 1.0 mL of 0.75 M sodium hydroxide was added to neutralise the hydrolised solution. Samples containing 1.0 mL of the resultant solution were taken and 1.0 mL: of DNSA reagent was added into each tube.

The tubes were mixed on a rotary stirrer and marbles were placed over the mouth of each in order to avoid evaporation,and then they were heated in a boiling water bath for 5 minutes.

The tubes were than transferred to an ice bath until cool and once cool each tube was washe out into a 25 mL volumetric flask and made up to the mark with distilled water.

The absorbance of the solutions were measured at 540 nm. Finally, the absorbances were plotted against the sucrose concentration.(fig. 2.9.2.). If the absorbance was greater than 0.7 a ten fold dilution was done.

2.9.3 - DETERMINATION OF SUCROSE DURING THE EXPERIMENTS

A 1.5 mL sample was taken from the fermenter vessel and the biomass was separated by centrifugation in an EPPENDORF 5413 centrifuge for 5 minutes.

Aliquots of supernatant 0.5 mL were placed into tubes containing 1.0 mL of 0.75 M hydrochloric acid and with marbles over the top of the tubes (to avoid evaporation), and the boiled in a water bath for 30 minutes and then neutralised with 1.0 mL of 0.75 M sodium hydroxide.

The resultant hydrolysed solution contained both glucose and fructose due to sucrose hydrolysis;

Acid hydrolysis

Sucrose ----------> Glucose + Fructose

The total reducing sugar concentration after hydrolysis was determined by the DNSA method. This method uses the reaction between reducing sugar and DNSA;

3,5 -dinitrosalycilic acid----> 3-amino 5 - nitrosalycilic acid.

This produces a colour change from yellow to red dark. This can be detected spectrophotometrically at 540 nm using a spectrophotometer (Unicam -SP 600 SERIES 2).

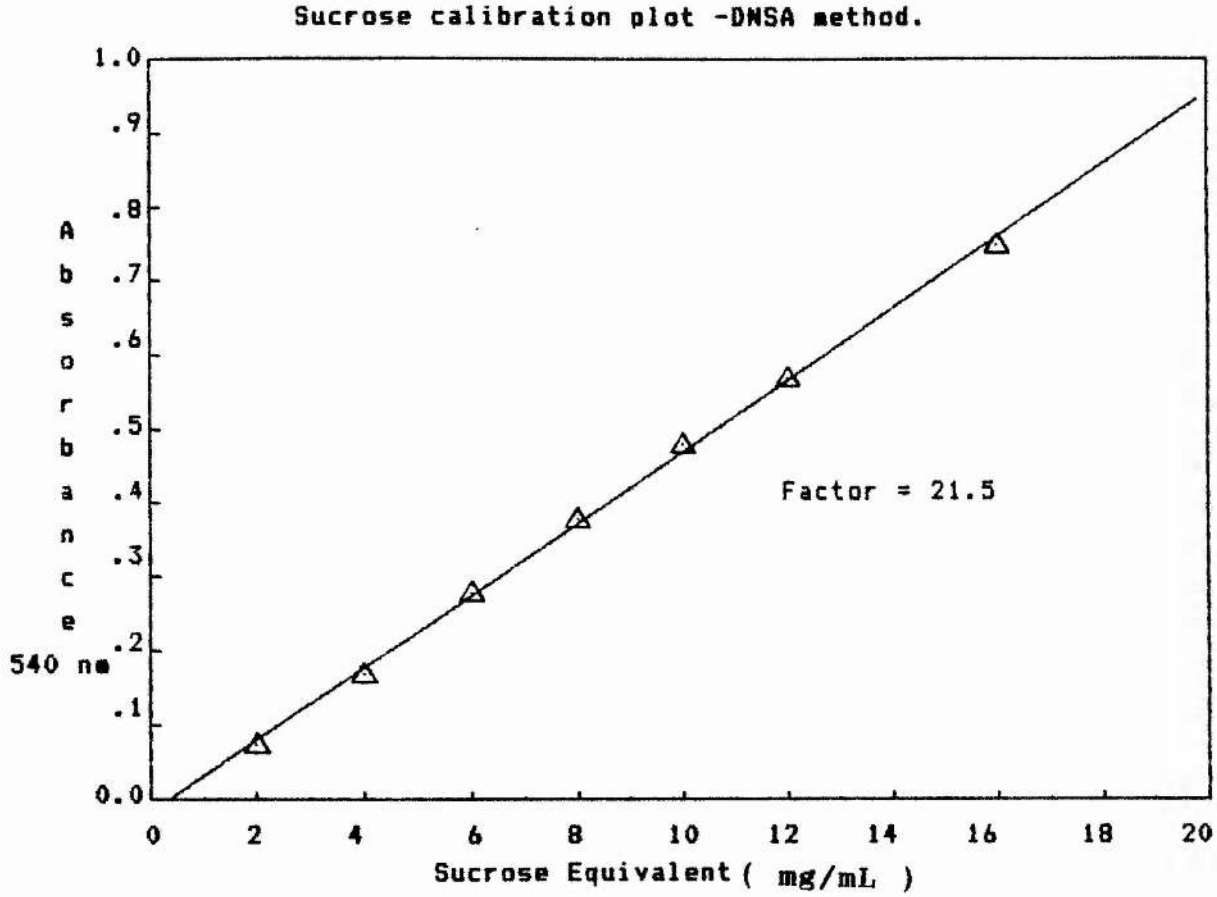


FIGURE 2.9.2 - CALIBRATION PLOT FOR DETERMINATION OF REDUCING SUGAR CONCENTRATION BY DNSA METHOD.

2.10 - DETERMINATION OF ETHANOL CONCENTRATION

Ethanol concentrations were determined by gas-liquid chromatography, using a PYE series 104 GLC apparatus (see section 2.4, h). The column was packed with Porapak Q 80 - 100 mesh and was maintained at 200 ^{o}C. The moving phase was nitrogen at a flow rate of 35 ml/min. Detection was made by a flame ionisation detector at 250 ^{o}C connected to a chart recorder.

A microsyringe was used to introduce samples into the column. Propanol was used as the internal standard, thus the ratio of the ethanol and propanol peaks were the features that were measured. As the propanol concentration was the same in all samples, these ratios were used to obtain the ethanol concentration by reference to a calibration plot.

2.10.1. - PREPARATION OF ETHANOL CALIBRATION PLOT

A series of aqueous standards were prepared containing ethanol in various concentrations from 1.0 to 9.0 mg per 1.0 mg of isopropanol. It was prepared as follows; Nine vials were set up each containing 0.1 mL of Isopropanol (10 mg/ml) to produce a final concentration of 1.0 mg/mL.

The ethanol concentration was increased from 1.0 to 9.0 mg/mL in successive vials by addition of ethanol in increments of 0.1 mL and the final volume in each vial was made up to 1.0 ml by addition of distilled water. Aliquots of 2 ul of each were run on the GLC apparatus three times each.

The ratio ethanol : isopropanol peak height was calculated and plotted against the respective ethanol concentration to produce a straight line calibration plot (Figure 2.10.1).

2.10.2. - ESTIMATION OF ETHANOL IN EXPERIMENTAL SAMPLES

Aliquots of fermentation medium (2.0 mL) were centrifuged to remove the cells and then 0.4 ml of supernatant were added to 0.1 mL of a standard solution of isopropanol (10 mg/mL) and then mixed. Aliquots of 2 uL from each sample were injected into the GLC and used to determine the ethanol concentration (g/L) obtained from an established calibration plot (see section 2.10.1).

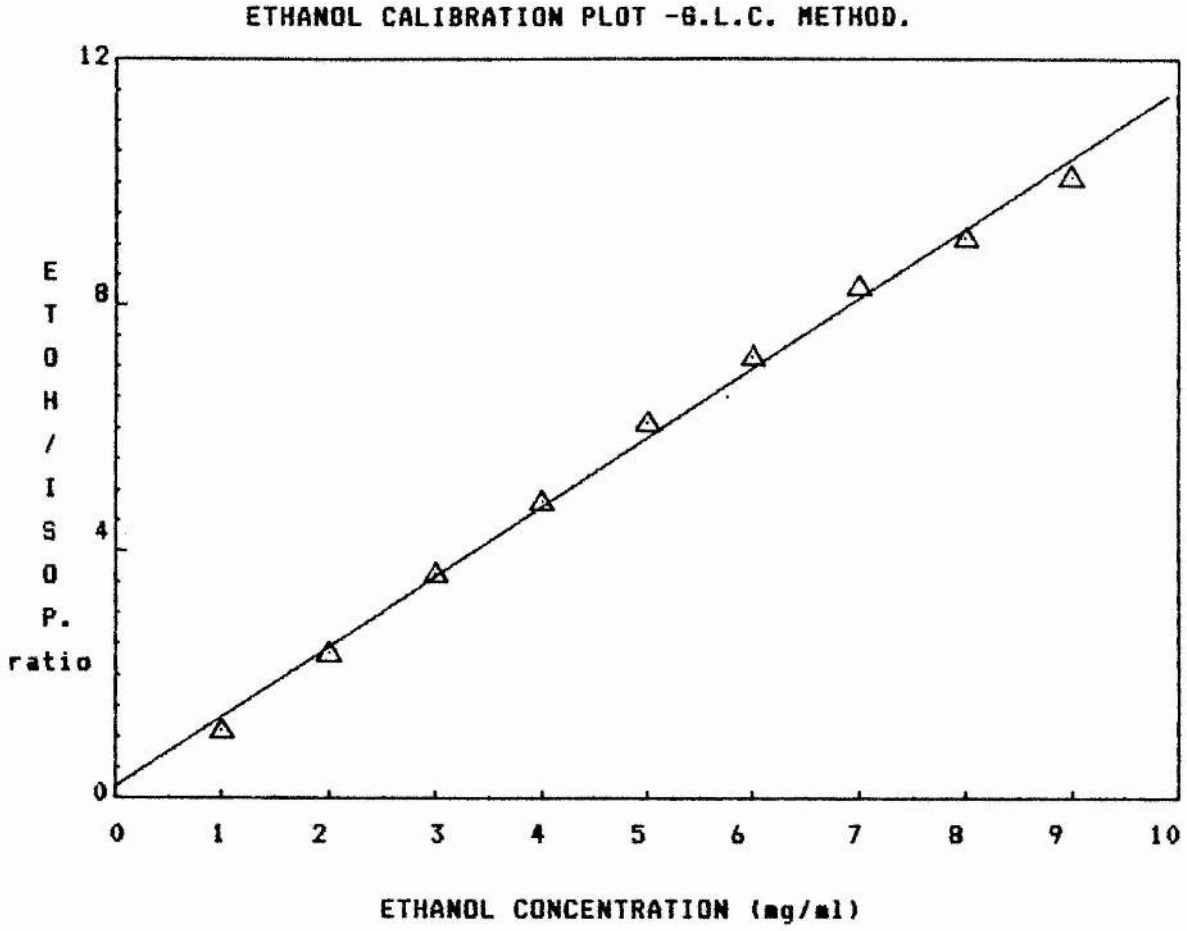


FIG. 2.10.1 - CALIBRATION PLOT FOR ETHANOL CONCENTRATION MEASUREMENTS

3 - RESULTS AND DISCUSSION

The growth of micro-organisms in any particular environment is governed by the restrictions that the environment imposes on its regulatory and metabolic processes. The ability of micro-organisms to adapt continually to changing conditions in nature is great.

In generated environments such as smaller laboratory fermenters the magnitude of changing conditions is reduced proportionally, since it is possible to achieve near to perfect environmental conditions as closely to a set point as desired.

Investigations into alternative yeasts strains capable of producing alcohol at higher temperature and ethanol levels has increased.

Fermentations processes which utilise renewable resources such as sugar cane juice, grain extracts, cellulose, starch and other polysacharides as substrates, have involved Saccharomyces cerevisiae and its related species.

The culture of strains of yeasts which exhibit significantly greater levels of ethanol and thermotolerance are seem as vitally important developments which would allow a more efficient utilisation of substrate.

In the last ten years, significant improvements in the technology available for both the agricultural and the industrial stages of ethanol production have been achieved [3,5,6,8,9,20,53,94,98]. An efficient fermentation of sucrose found in the sugar cane juice and molasses is a key to the economic production of ethanol from these raw materials in a country like Brazil.

The following sections describe the results of experiments carried out with the aims of optimizing ethanol production at high temperatures by *Saccharomyces cerevisiae* (PLA 851) in a chemostat culture with feedback of biomass.

The selection of the strain was based on previous batch tests and the results are the average parameters evaluated from experimental data.

The continuous experiments were performed in a chemostat with feedback of biomass in order to evaluate the effects of changing temperatures, flow rates and substrate concentrations on the production of ethanol by Saccharomyces cerevisiae (PLA 851).

3.1 - SELECTION OF APPROPRIATE STRAINS FROM BATCH DATA ANALYSIS.

In order to select an appropriate strain to proceed with the continuous culture experiments, two *Saccharomyces cerevisiae* (42-F and PLA 851), and *Saccharomyces boulardii* (IZ 1904) strains which are all widely used in the Brazilian Alcohol fermentation industry, were batch cultured and some of their kinetic parameters determined at temperatures of 30^{o}, 35^{o}, and 40^{o} C.

Table 3.1 shows the kinetic parameters analysed through these curves. The maximum specific growth rates were determined as described in 2.7. As can be seen in table 3.1 the values of μ_{max} decreased as the temperature increased.

Our data are in agreement with that of van Uden[27], who pointed out that when a population of *Saccharomyces cerevisiae* is transferred to a liquid stirred medium incubated at a temperature between the optimum and the maximum, the specific growth rate decreases with increasing temperature. However, *Saccharomyces cerevisiae* (PLA 851) showed higher values of μ_{max} for each temperature tested, compared to 42-F and IZ 1904 strains.

Micro organism	T ^{o}C	μ_{max} h^{-1}	x g/l	q_s (g/g.h)	q_p	$Y_{p/s}$
Sacch.	30	.26	.91	3.5	1.3	.37
cerevisiae	35	.21	.76	4.7	1.5	.32
F -42	40	.08	.30	2.6	1.0	.38
Sacch.	30	.23	1.3	3.3	1.6	.48
boulardii	35	.18	1.2	3.6	1.7	.47
IZ 1904	40	.12	0.5	1.2	0.6	.50
Sacch.	30	.42	1.5	3.0	1.6	.53
cerevisiae	35	.38	1.3	4.7	2.2	.47
PLA 851	40	.29	0.7	2.4	1.3	.54

TABLE 3.1 - SHOWING THE EFFECT OF TEMPERATURE ON THE SPECIFIC GROWTH RATE, ETHANOL YIELD, SPECIFIC SUCROSE UPTAKE AND ETHANOL PRODUCTION RATES AND BIOMASS IN A 2 % SUCROSE MEDIUM ON THE THREE SACCHAROMYCES ALCOHOL - PRODUCING STRAINS.

The specific rate of substrate consumption (q_s) in a culture is given by :

$$q_s = ds/dt \; . \; 1/x$$

Where x is the biomass concentration.

The three strains showed higher values of sucrose uptake at 35 ^{o}C. The *Saccharomyces cerevisiae* (PLA 851) strain showed the highest values for each temperature compared to the other strains.

The ratio of cells produced to substrate consumed is defined as yield coefficient (Y). Generally, yield coefficients are used to characterize fermentation processes. However, they depend on biological parameters (O_2, C/N ratio and P content of the medium).

The productivity of a culture system may be described as the output of biomass or product per unit of time of the fermentation. Thus, the productivity of a batch culture may be represented as;

$$R_{batch} = (x_{max} - x_o)/(t_i + t_{ii})$$

Where R_{batch} = Output of a culture in terms of cell or product concentration per hour.

x_{max} = Maximum cell or product concentration achieved.

x_o = Initial cell or product at inoculation.

T_i = The time during which the organism grows at μ_{max}.

T_{ii} = Is the time during which the organism is not growing at μ_{max}, including time for setting - up the process[11].

According to the yields results presented in Table 3.1, *Saccharomyces cerevisiae* PLA 851 showed the highest yield value ($Y_{p/s}$ = 0.54) at 40 ^{o}C. Then, this strain was chosen for the experiments in continuous culture with and without feedback of biomass.

Figures 3.1 (a), (b) and (c) shows the growth curves for biomass production, sucrose consumption and ethanol production by Saccaromyces cerevisiae PLA 851 at different temperatures.

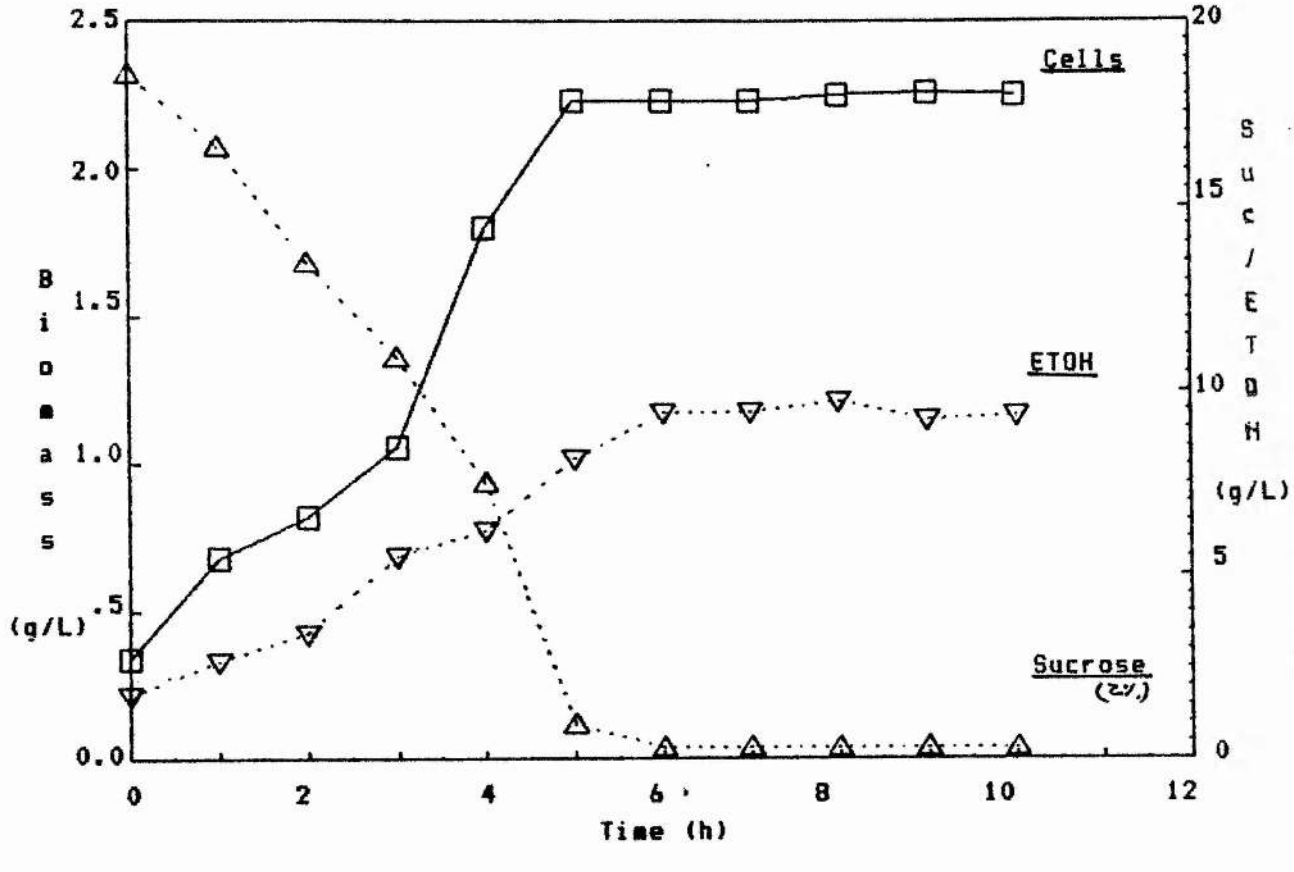


FIGURE 3.1. a - GROWTH CURVE OF SACCHAROMYCES CEREVISIAE (PLA 851) IN A BATCH EXPERIMENT AT 30 °C ON 2 % SUCROSE MEDIUM.

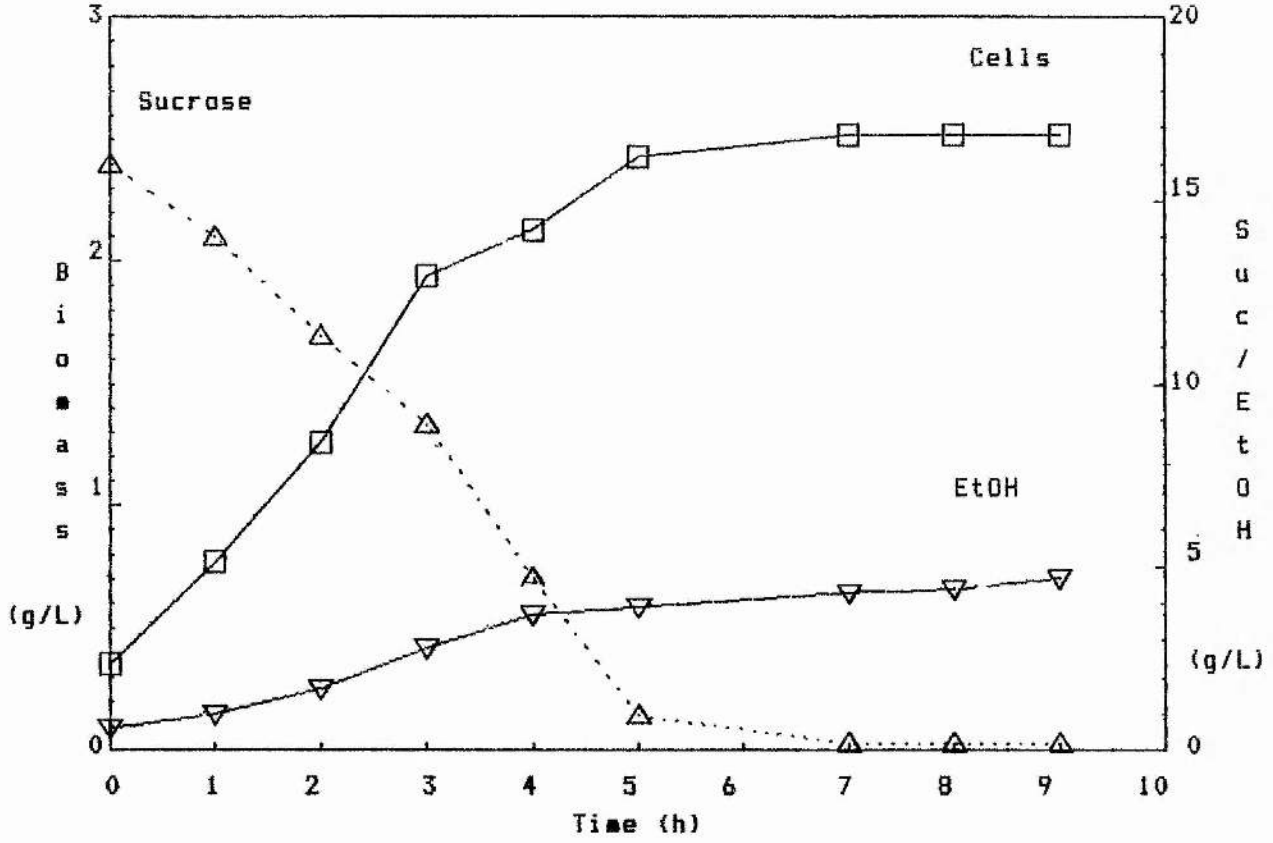


FIGURE 3.1. b - GROWTH CURVE OF SACCHAROMYCES CEREVISIAE (PLA 851) IN A BATCH EXPERIMENT AT 35 ^{o}C ON 2% SUCROSE MEDIUM.

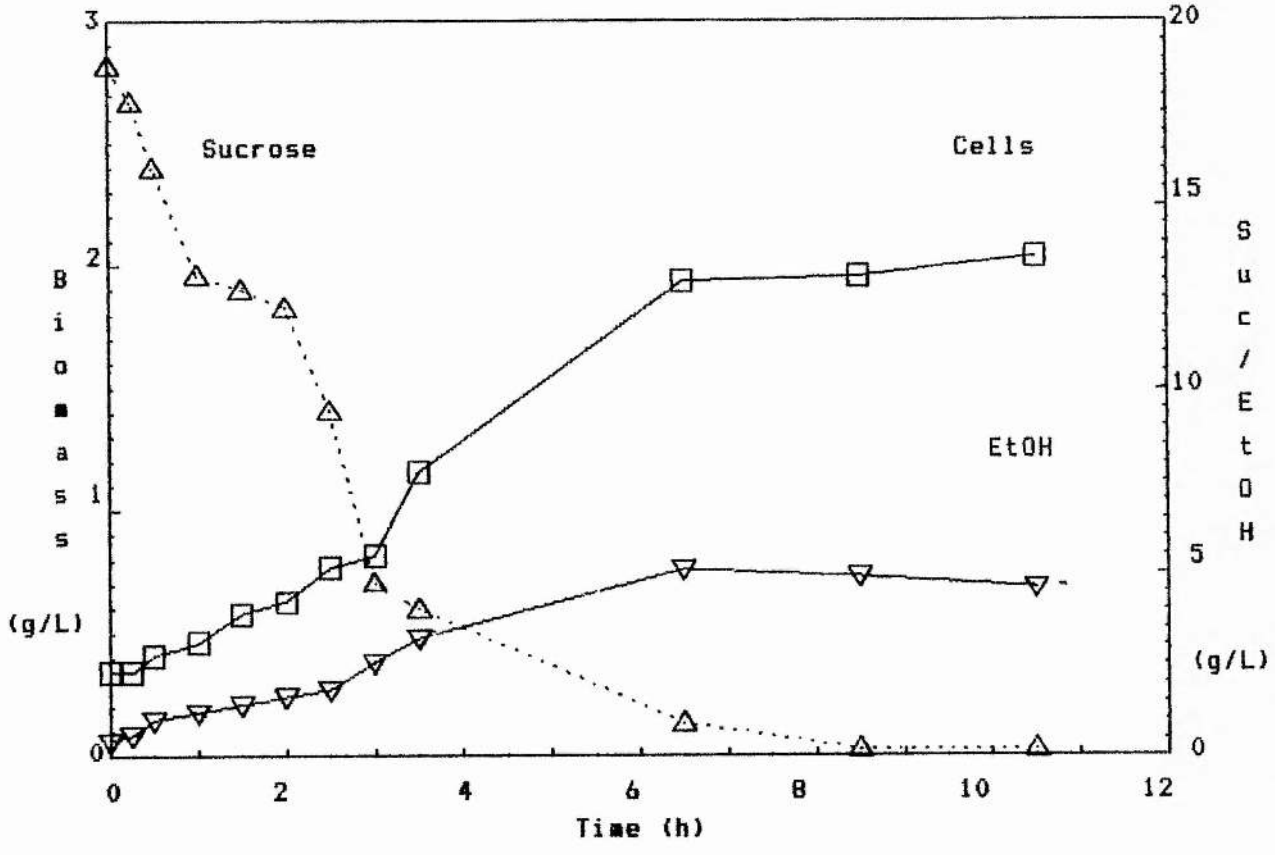


FIGURE 3.1. c - GROWTH CURVE OF SACCHAROMYCES CEREVISIAE (PLA 851) IN A BATCH EXPERIMENT AT 40 ^{o}C ON 2 % SUCROSE MEDIUM.

Alternatively, the slope of the tangent of the product formation curve is a measurement of maximal productivity.

This method has been used in our experiments. The specific rate of production, q_p, is given by the following equation :

$$q_p = (dp/dt)/x$$

For the three strains the maximal ethanol production rate was achieved at 35 ^{o}C, falling at 40 ^{o}C.

The ethanol yields of several species of yeast have been tested and shown to decrease with increasing fermentation temperature[20,25.35,41]. However, the presence of sterols seems to reduce the disordering effects of elevated temperatures on the fluidity membranes [41].

A larger number of temperature - sensitive cellular constituents or processes have been detected in a wide variety of yeast[26,39,46,62,86,87].

3.2 - DESIGN AND OPERATION OF THE CHEMOSTAT WITH FEEDBACK OF BIOMASS.

The chemostat with feedback of biomass used in our experiments was based on the model (d), see section 1.2.4. The figure 3.2 (a), shows the diagrammatic representation of the chemostat with feedback of biomass.

The chemostat consists of two reservoirs (1, 7) with 20 liters capacity; one fermenter glass vessel of 1 Liter capacity with 690 mL working volume (3); two peristaltic pumps (2, 6); one sedimentation vessel with working volume of 110 mL; and a Citenco motor drive (4) powering a magnetic stirrer providing agitation and aeration.

The figure 3.2 (b) and 3.2 (c) shows the apparatus constructed with angle-iron frames, supporting the main parts of the chemostat, that is, the fermenter vessel, motor drive, peristaltic pumps and the sedimentation vessel.

The chemostat was covered by polythene sheets in order to keep the temperature constant in the system as a whole .

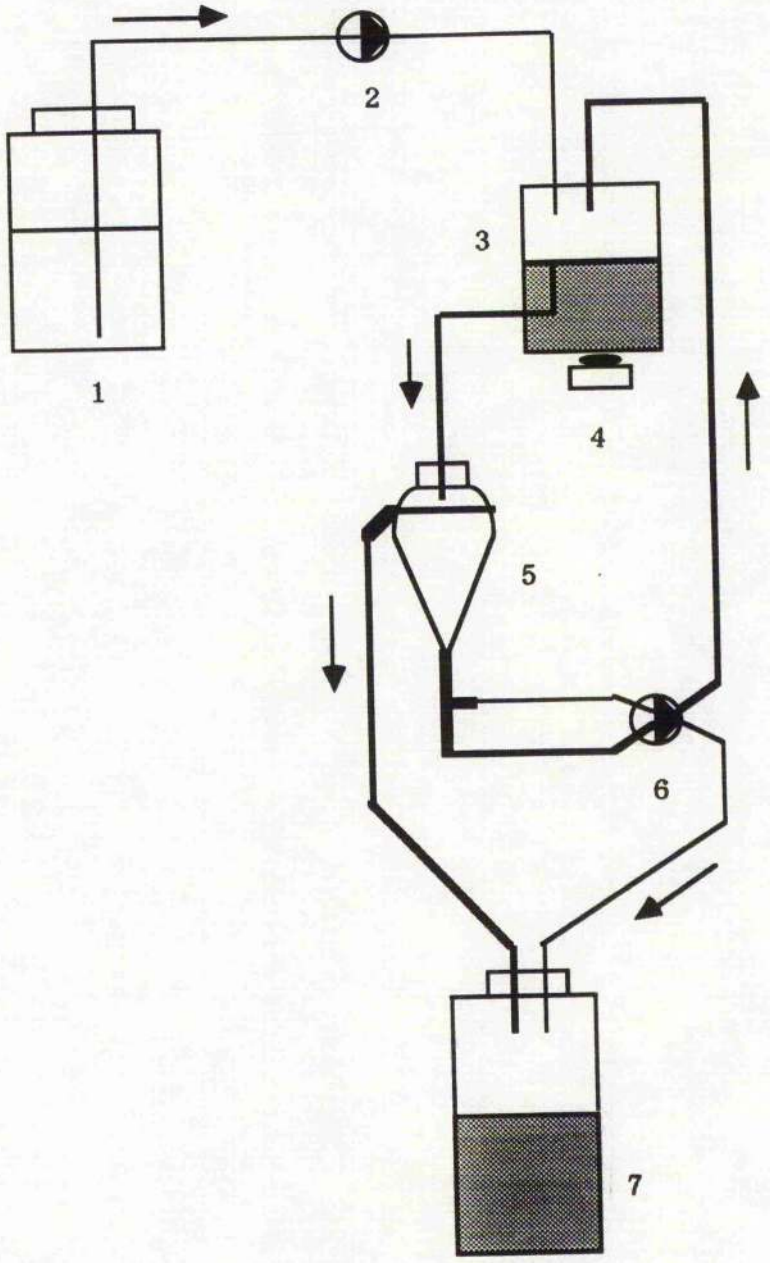


FIGURE 3.2 (a): SCHEMATIC DIAGRAM OF THE CONTINUOUS STIRRED REACTOR WITH RECYCLE OF BIOMASS.

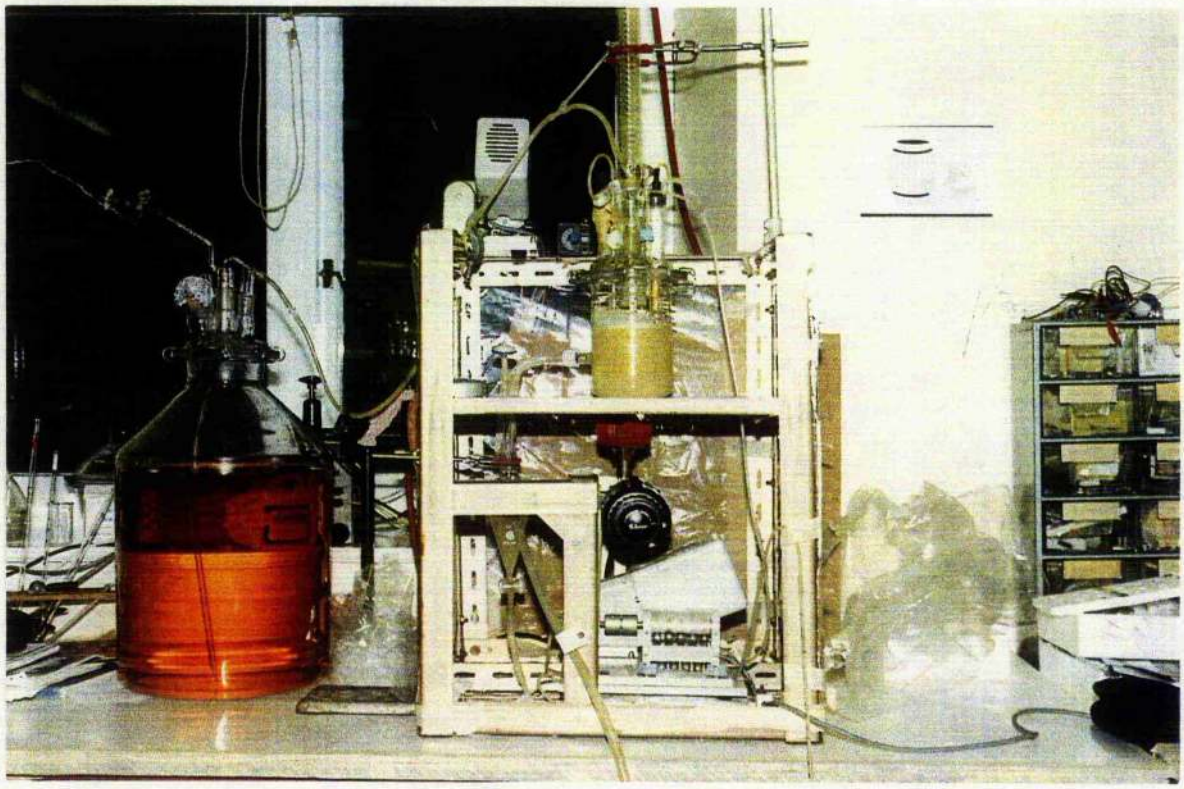


Figure 3.2 (b): THE APPARATUS USED IN THE CONTINUOUS CULTURE WITH FEEDBACK OF BIOMASS.

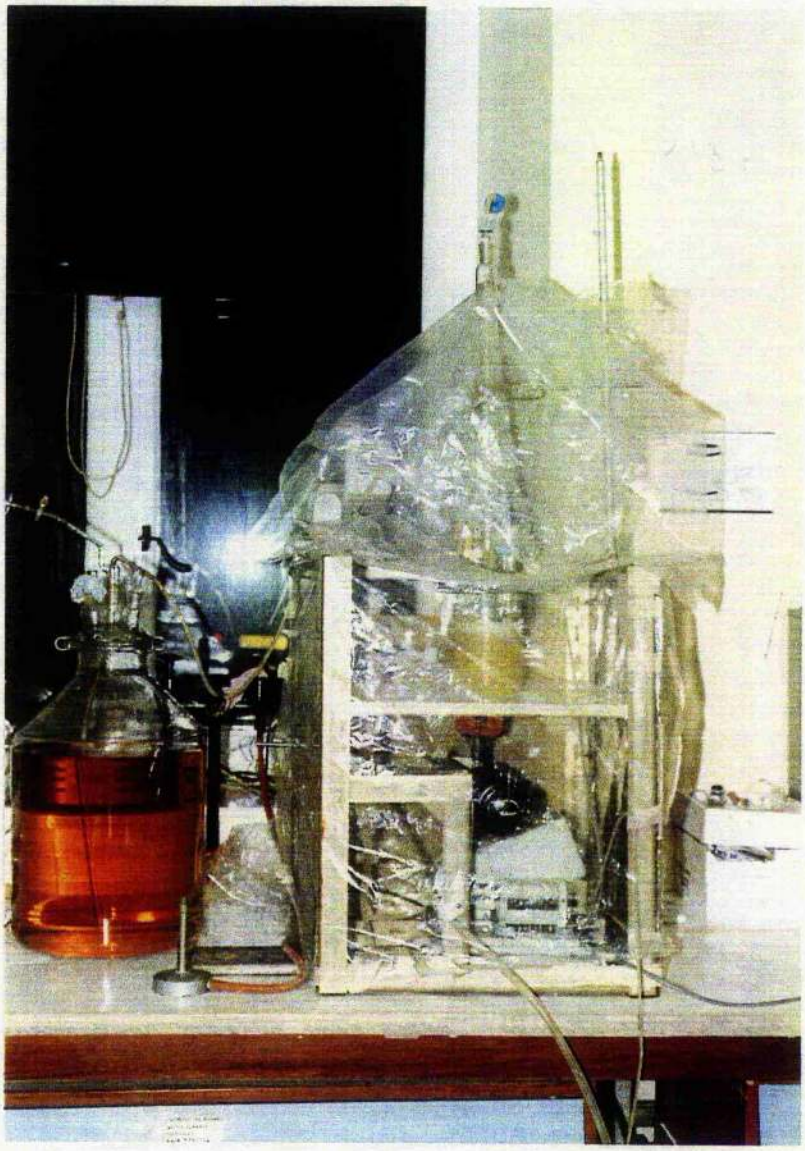


Figure 3.2 (c): SHOWS THE POLYTHENE SHEETS COVERING THE APPARATUS TO KEEP THE TEMPERATURE CONSTANT.

In most microbiological processes, foaming is a problem. The commonest cause is due to proteins in the medium, which may denature at the air-broth interface and form a skin which does not rupture readily.

The foaming can cause removal of cells from the medium which will lead to autolysis and the further release of microbial cell proteins will probably increase the stability of the foam. If uncontrolled then the air-filter exits of the fermenter become wet and there is danger of microbial infection and the possibility of siphoning leading to loss of product.

Foaming is normally controlled by either chemical or mechanical means. Chemical antifoams are surface active agents which reduce the surface tension in the foams. However, the concentration of many antifoams which are necessary to control fermentation will reduce the oxygen-transfer rate by as much as 50 %, therefore antifoam additions must be kept to an absolute minimum[93].

In our experiments a mechanical foam breaker device was used instead of chemical antifoams. It was developed in our laboratory and fitted on the top of the pipe mouth drain tube inside of the fermenter vessel. It is formed by a teflon ring, a borosilicate tube and three wire support held in place with silicone sleeves, see figure 3.2 (d).

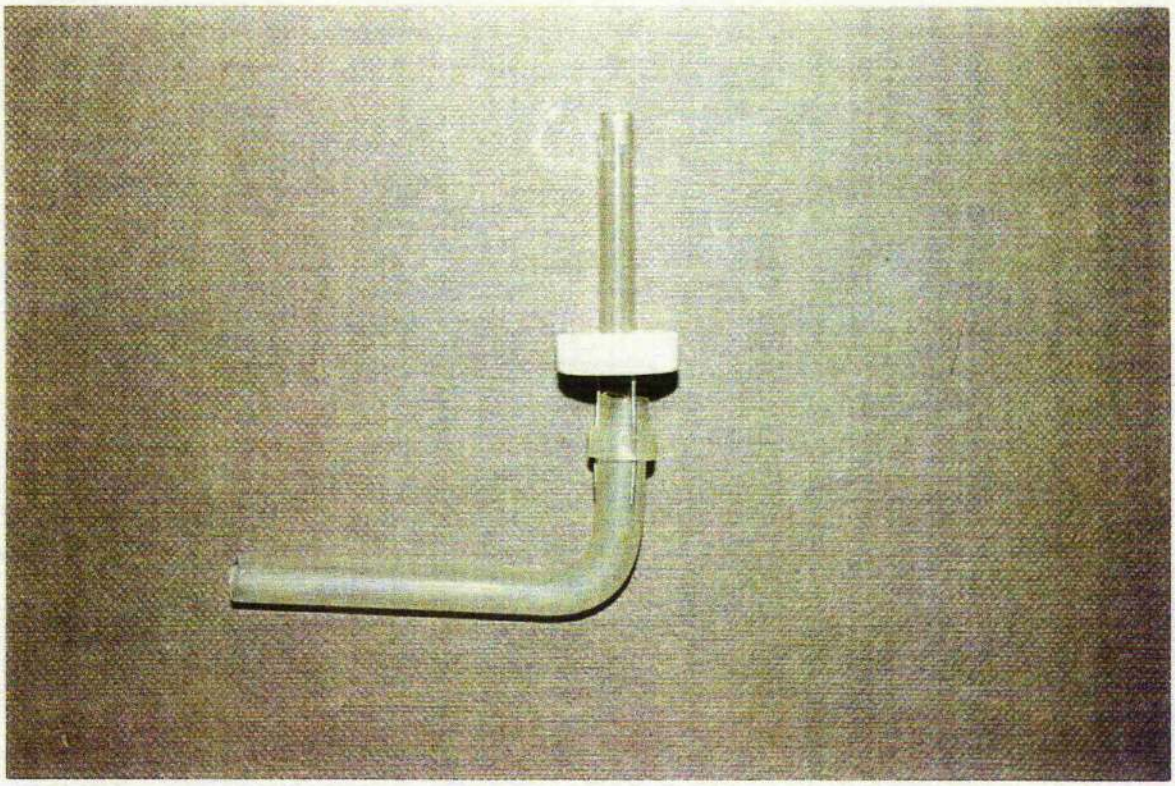


Figure 3.2 (d): SHOWS THE MECHANICAL ANTI-FOAM BREAKER DEVICE.

The high degree of aeration and agitation frequently gives rise to the undesirable phenomenon of foam formation. In extreme circumstances the foam may overflow from the fermenter via air outlet or sample line resulting in the loss of medium and product, as well as increasing the risk of contamination.

The effects of that mechanical antifoam breaker device was observed during continuous experiments at high temperatures. The increases in the temperature of fermentation reduced the phenomenon of foam formation (decreasing the viscosity) and it also helped us to maintain the control over the outflow rate from the fermenter to the sedimentation vessel, see figure 3.2 (e) and (f).

The continuous culture experiments with and without feedback were carried out under the conditions described in section 2.5.2. When the cells achieved late exponential phase in batch culture, the continuous influx of medium was started. The flow rate was determined measuring the time (min) to fill a 10 mL volumetric vessel with culture. The dilution rates were calculated dividing the flow rate by the volume of culture inside of the fermenter (690 mL).

Each experiment started at 30 ^{o}C. After a steady state had been achieved samples started to be collected every 8 or 12 hours.

The temperature or dilution rate were changed after approximately 100 hours of experiment.

FIGURE 3.2 (e): SHOWS THE MECHANICAL ANTI-FOAM BREAKER DEVICE INSIDE OF THE FERMENTER VESSEL.

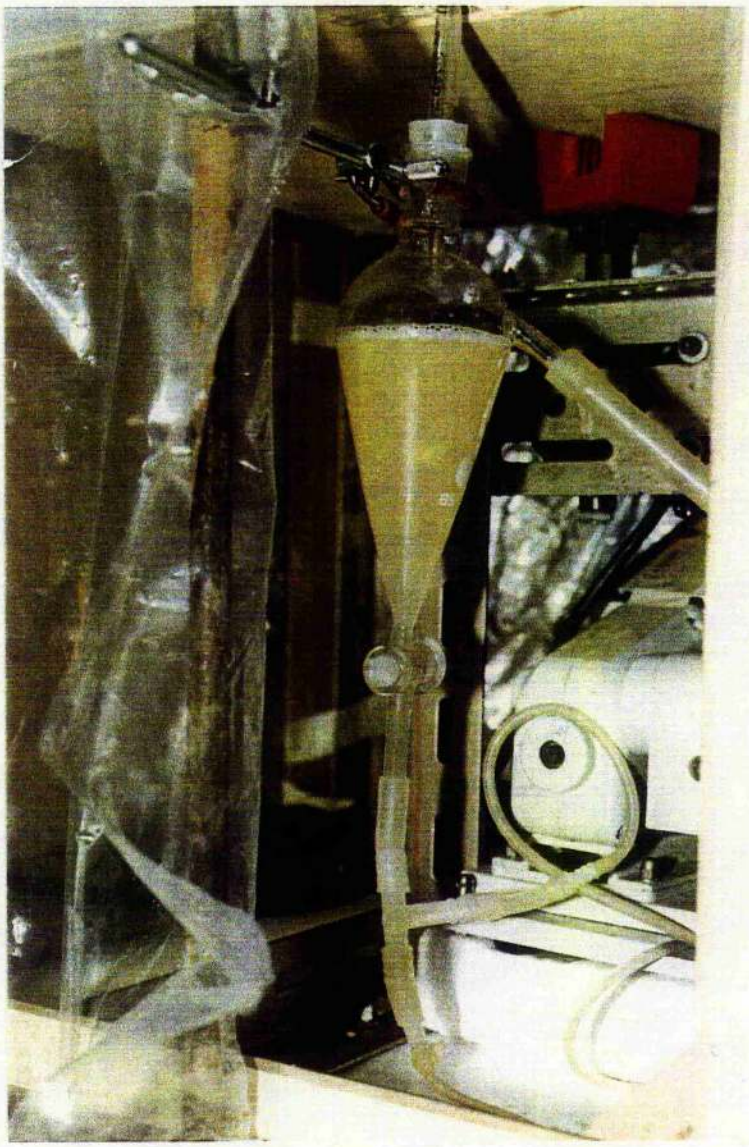


FIGURE 3.2 (f): SHOWS THE SEDIMENTATION VESSEL WITH A "T" CONNECTION TUBE USED TO RECYCLING THE CONCENTRATED BIOMASS.

For the continuous experiments with feedback of biomass, the proceedings were similar, but after a steady state had been achieved the concentrator vessel was connected to the main system.

The working volume to calculate the dilution rate in this case, includes the volume inside of the concentrator (110 mL). The dilution rates were 0.05, 0.10, 0.15, 0.20 and 0.25 h^{-1}.

3.3 - GROWTH AND ETHANOL PRODUCTIVITY UNDER CONTINUOUS CULTURE WITH AND WITHOUT FEEDBACK AT TEMPERATURE OF 30, 35 AND 40 ^{o}C.

The table 3.3 a and b, shows some kinetic parameters from *Saccharomyces cerevisiae* PLA 851 growing with and without recycling of cells, on 2 % sucrose medium at temperatures of 30, 35 and 40 ^{o}C.

The dilution rate for the experiments without feedback was 0.10 h^{-1}, below the μ_{max} detected (0.29 h^{-1}) for the strain under batch mode at 40 ^{o}C.

FERMENTATION PARAMETERS

T	D	x	s	p	a	g	b	μ	P_{cont}	Osc.Freq
^{o}C	h^{-1}	g/l	g/l	g/l				h^{-1}	g/l.h	(μHz)
30	0.10	.72	nd	6.1	-	-	-	0.10	.61	8.7
35	0.10	.70	nd	5.4	-	-	-	0.10	.55	8.7
40	0.10	.42	2.7	3.2	-	-	-	0.10	.32	4.0

T = temperature (^{o}C)

D = dilution rate (h^{-1})

x = biomass in steady state (g/l)

s = average sucrose conc. in steady state (g/l)

p = average ethanol conc. in steady state (g/l)

nd = not - detected

μ = growth rate (h^{-1})

P_{cont} = productivity (g/l.h)

Osc.Freq = Oscillation frequency (μHz)

(Ethanol concentration).

TABLE 3.3 a - Shows the kinetic parameters from Saccharomyces cerevisiae PLA 851 in continuous culture without feedback of biomass in 2% sucrose medium at different temperatures.

FERMENTATION PARAMETERS

T	D	x	s	p	a	g	B	μ	P_{cont}	Osc.freq
^{o}C	h^{-1}	g/l	g/l	g/l				h^{-1}	g/l.h	(μHz)
30	.10	2.1	nd	10.2	.157	1.4	.92	.09	1.0	3.5
35	.10	1.9	nd	11.2	.157	1.5	.91	.09	1.1	3.5
40	.10	1.8	nd	11.9	.157	1.8	.85	.08	1.2	5.0

T = temperature (^{o}C)

D = dilution rate (h^{-1})

x = biomass in steady state (g/l)

s = average sucrose conc. in steady state (g/l)

p = average ethanol conc. in steady state (g/l)

a,g and B = dimensionless constants

nd = not - detected

u = growth rate (h^{-1})

P_{cont} = productivity (g/l.h)

Osc. freq = Oscillation frequency (μHz)

(Ethanol concentration).

TABLE 3.3 b - Shows kinetic parameters from Saccharomyces cerevisiae PLA 851 in continuous culture with feedback of biomass on 2 % sucrose medium at different temperatures.

The continuous culture technique enables an analysis of physiological states of organisms under steady state conditions when nutrient concentration remains constant for a high number of generations. However, because the environmental conditions that prevail in a continuous flow system are quite different from those in closed system cultures, microorganisms may express unexpected features of behaviour [93].

Figures 3.3 (a) and (b), shows the results of the experiments without and with feedback of biomass. Under conditions of no feedback (B = 1), sustained oscillations in both ethanol concentration and biomass were observed. Ethanol concentrations at temperatures of 30 and 35 ^{o}C oscillated with a frequency of 8.7 microhertz dropping to 4.0 microhertz at 40 ^{o}C.

Amplitudes of oscillation were of the order of 2.0 g/l. Biomass also oscillated with much the same frequency but was much more limited in amplitude (< 0.1 g/l).

Under conditions of feedback (B = 0.9), sustained oscillations in ethanol concentration were also observed but at a lower frequency of 3.5 microhertz at 30 and 35 ^{o}C rising slightly to 5.0 microhertz at 40 ^{o}C.

Amplitudes of ethanol oscillations were of the same order as under no feedback conditions but biomass amplitudes were of a much greater order (1 g/l). A considerable productivity increase was observed under feedback conditions rising from 0.32 g/l.h to 1.2 g/l.h at 40 ^{o}C.

Borzani et al.[94] found oscillations of low amplitude for the cells and the substrate concentrations working with *Saccharomyces cerevisiae* growing under aerobic and anaerobic continuous culture. However, they observed that the average yield coefficients during the transient states were equal to the yield coefficient obtained during steady state experiments.

Oscillations have also been reported in chemostat cultures of *Saccharomyces cerevisiae* by Porro et al.[95]. The authors reported that the period of oscillations seemed to be related to the mass doubling time, showing a relationship with the parent cells and daughter cells generation time. They concluded that the oscillations were related to a condition of growth that does not allow a full metabolism of glucose during specific phases of cell cycle, such as the bud emergence.

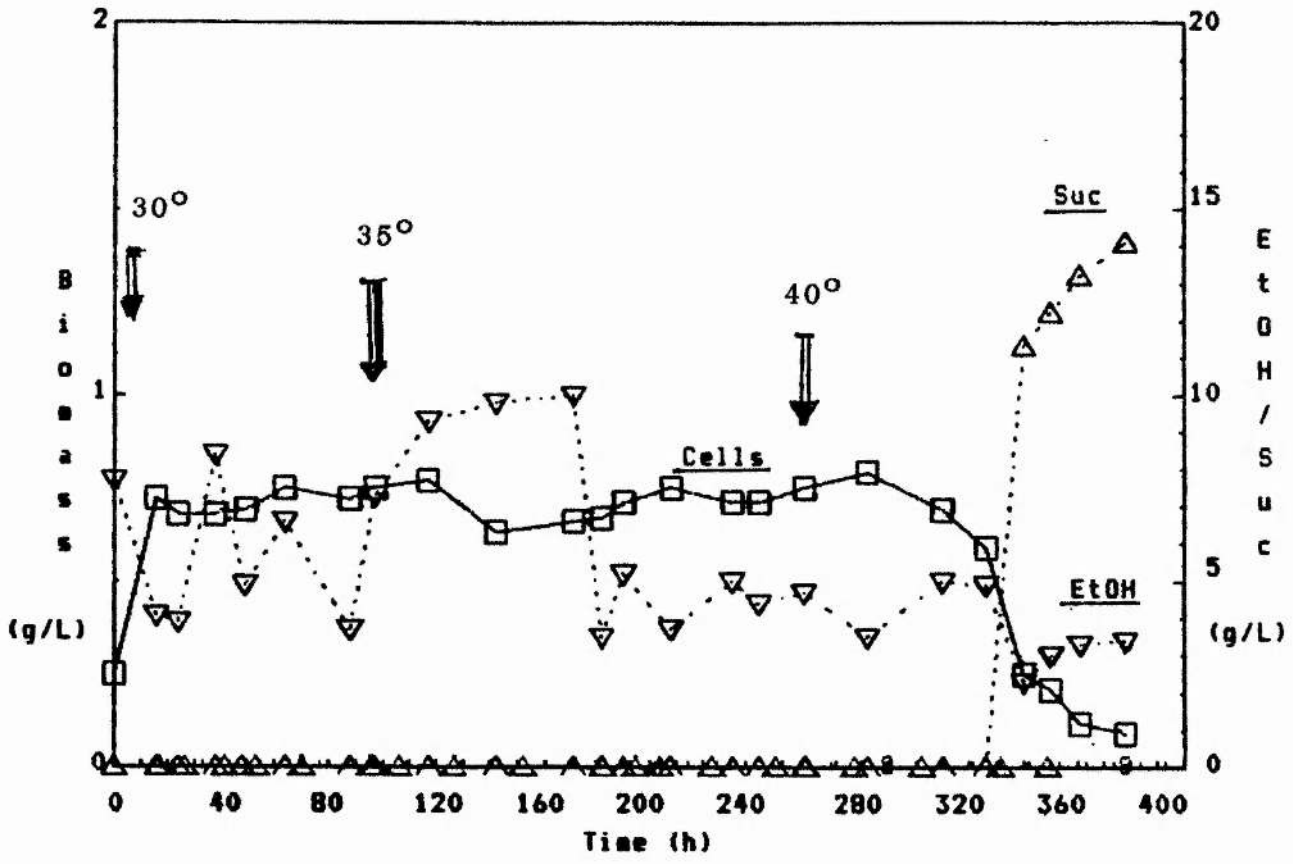


FIGURE 3.3 a - Shows the results of a Continuous culture experiment without feedback of biomass on 2 % sucrose medium at D = 0.10 h^{-1} at different temperatures.

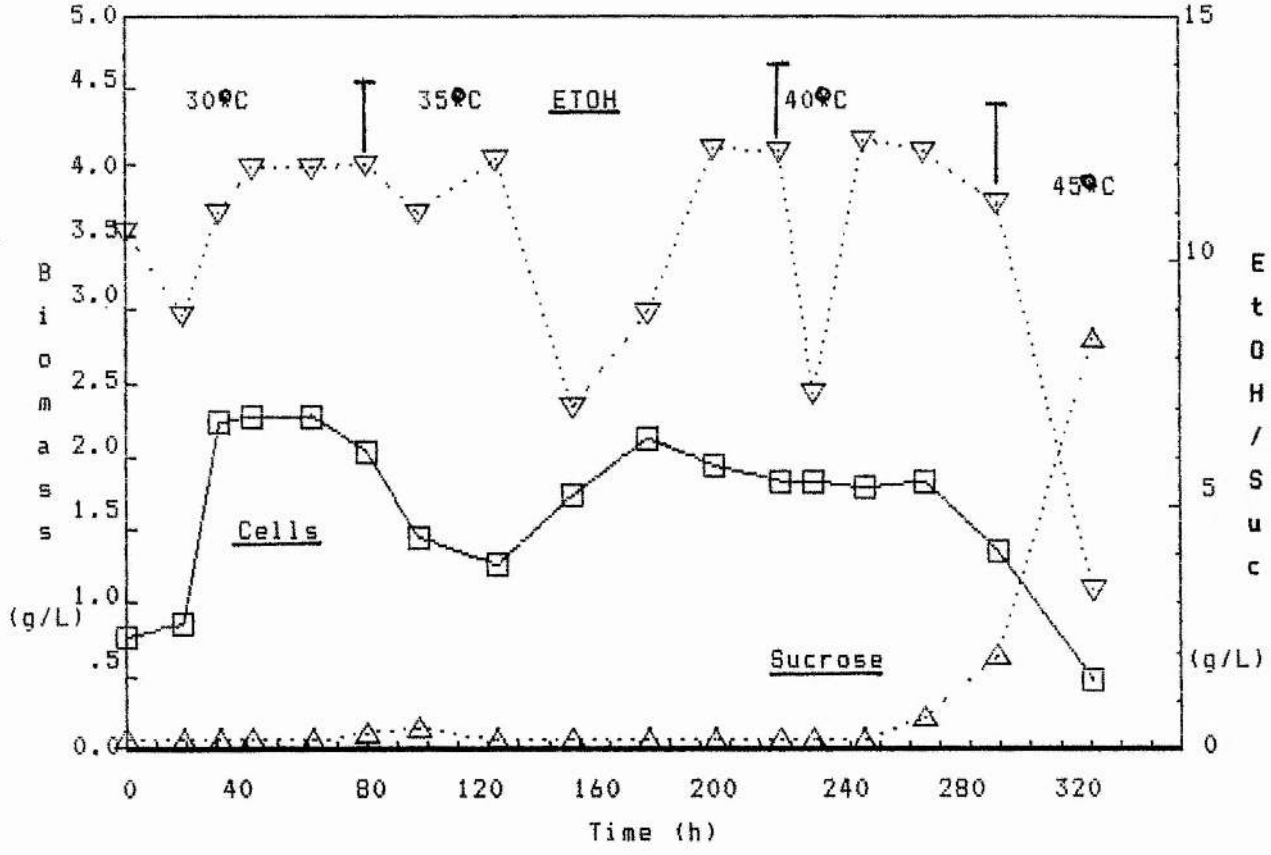


FIGURE 3.3 b - Shows the results of a Continuous culture experiment with feedback of biomass on 2% sucrose medium at D = 0.10 h^{-1} at different temperatures.

The complex changes of the cell population were demonstrated by continuous and periodic modification of cell volume and protein distribution. Eventhough, the researchers did not work under anaerobic conditions, the explanation might be extended to the oscillations found for ethanol production.

For the ethanol productivity calculations p can be used instead of x. However, in laboratory experiments the productivity can be obtained as the product of D x p.

We can see that the ethanol productivity without feedback was about 50 % of the productivity with cell recycling for the temperatures of 30 and 35 ^{o}C. At 40 ^{o}C, the productivity without feedback was only 30 % compared to the productivity with feedback of biomass.

Biomass growth in continuous culture is limited by the amount of substrate. A fall in the biomass concentration will be associated with a rise in the substrate concentration. This is consistent with our results at 40 ^{o}C without feedback.

The high temperature led to a fall in the biomass level with a rise in the residual sucrose concentration and a decrease in the ethanol production.

The productivity of a continuous fermentation might be limited by the low concentration of yeast cells in the fermenter. As it has already been said, the dilution rate must be less than the maximum specific growth rate. However, by separating a fraction of cells from the culture and returning then to the fermenter this limitation can be overcome [96].

According to the Kinetics of chemostat with external feedback of biomass, in the steady - state $\mu = B.D$ where $0 < B < 1$, which enables the chemostat to be operated at dilution rates greater than u_{max}.

The B factor is calculated from the equation :

$$B = (1 - ag)/(1 - a)$$

Where (g) is the ratio of the biomass concentration in the fermenter to the biomass in the concentrated stream, and (a) is expressed by the equation below :

$$a = (F_s - F)/(F_s)$$

Where F is the feed stream volumetric flow rate, and F_s is the bleed stream flow rate.

3.4 - EFFECT OF DILUTION RATE ON THE OUTCOME OF CHEMOSTAT OPERATIONS WITH FEEDBACK OF BIOMASS AT 40 ^{o}C.

The Table 3.4 shows the effect of dilution rate on the productivity of *Saccharomyces cerevisiae* PLA 851 strain growing on 2, 5, 10 and 20 % sucrose medium at 40 ^{o}C.

The productivity values increased with D for all sucrose concentrations.

The highest productivities were found at D = 0.25 h^{-1} for both 10 and 20 % initial sucrose concentration respectively, 5.6 and 4.7 (g/l.h).

At this dilution rate (D = 0.25 h^{-1}) the effect of temperature on the specific growth rate becomes evident.

Similar productivities were observed for 5 and 20 % initial sucrose concentration at D = 0.05 h^{-1}. The highest value of ethanol productivity also ocurred at D = 0.20 h^{-1} (4.3 g/l.h). At temperatures higher than the optimum (30 ^{o}C for yeast) the thermal death rate is increased and the critical dilution rate (which is related to u_{max}) is decreased.

FERMENTATION PRODUCTIVITIES (g/L.h)				
Dilution rate (h^{-1})	SUCROSE CONCENTRATION (%)			
	2	5	10	20
0.05	0.6	1.4	1.6	1.4
0.10	1.1	1.8	2.4	2.5
0.15	1.5	2.0	2.8	2.5
0.20	1.9	2.2	3.1	4.3
0.25	2.0	2.9	5.6	4.7

TABLE 3.4 - Productivity of Saccharomyces cerevisiae (PLA 851) in a media with 2, 5, 10 and 20 % sucrose as carbon source at 40 ^{o}C in continuous culture experiment with feedback of biomass at different dilution rates.

A mathematical model for glucose to ethanol fermentation at high yeast cell concentration was developed by Lee et al.[96]

According to the model, the B/F ratio is the ratio of bleed stream volumetric flow rate (which corresponds to the F_s from the kinetic equation) to feed stream volumetric flow rate.

A B/F = 1 occurs when there is no cell recycling.

As the B/F ratio is decreased the dilution rate can be increased while still maintaining the same out put concentration of product.

Since productivity can also be described as the product of the dilution rate and the product concentration, decreasing B/F increases productivity. However, the simulation conditions are that the bleed stream rate is held constant, but the feed rate increased. That would lead to increasing in the cell concentration of the fermenter.

Our experience showed that a very high concentration of cells in the bleed stream imposes a heavy burden on the cell separation unit causing frequent failures.

We found that the specific growth rate which is the product of D and B would be more realistically related to the productivity of the system concerned to biomass and ethanol production per unit volume and unit time.

3.5 ENHANCEMENT OF THERMAL TOLERANCE IN S.CEREVISIAE BY PREINCUBATION AT HIGH TEMPERATURE.

The *Saccharomyces cerevisiae* PLA 851 strain was grown on 2 % sucrose medium at D = 0.10 h^{-1} and initial temperature of 30 ^{o}C. After a steady-state had been achieved, the temperature was increased to 35 ^{o}C and subsequentially to 40 ^{o}C and 45 ^{o}C.

The biomass concentration decreased with increasing temperature. However, a significant fall was detected at 45 ^{o}C, and the temperature was reset to 40 ^{o}C again to prevent washout.

As the response to exposure to a high temperature (45 ^{o}C), the biomass level which was about 1.8 g/L in the first run at 40 ^{o}C, now achieved levels of 2.7 g/L.

As can be seen in Figure 3.5, even at 40 ^{o}C (after preincubation at 45 ^{o}C), the biomass level was about 2.7 g/L, higher than the level found at 35 ^{o}C (1.6 g/L) without preincubation.

It is noticeable, however, that the average ethanol concentration only increased very slightly indicating a change in cellular metabolism.

The simultaneous enhancement of ethanol and thermal tolerance in *Saccharomyces cerevisiae* has been shown to occur after a short preincubation time at high temperature[19].

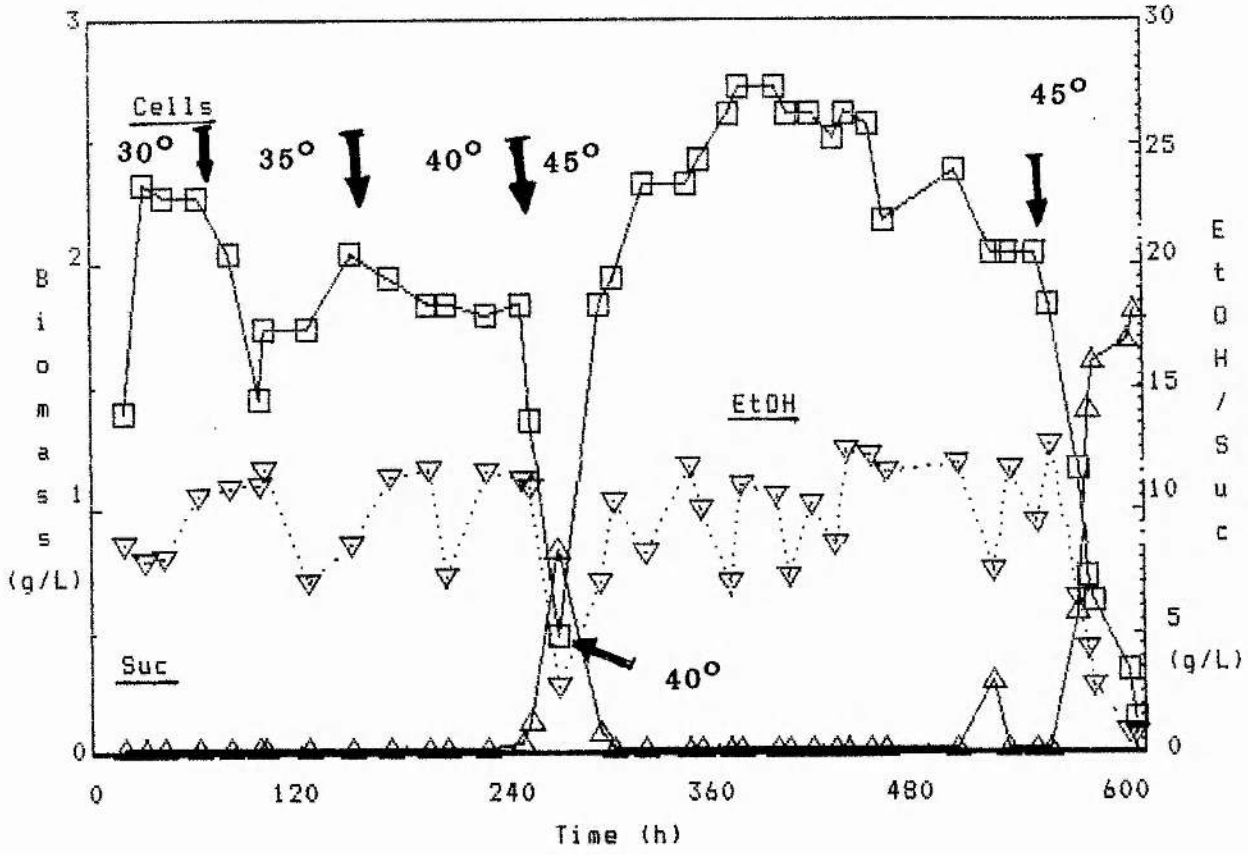


FIGURE 3.5 - Ethanol and biomass production (2 % Sucrose medium) at increasing temperature from 30 to 40 °C and illustrating the effect of " heat shocking " at 45 °C on subsequent biomass production at 40 °C.

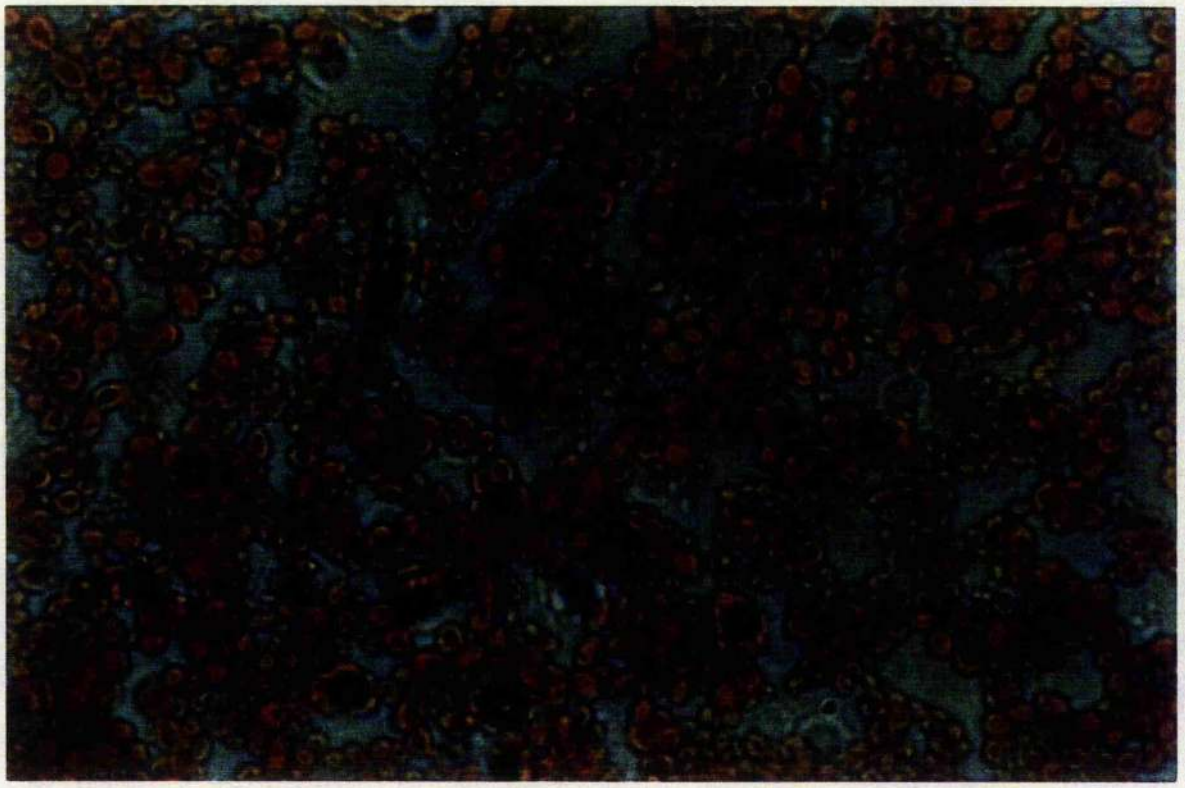
Increases in temperature and ethanol concentration have shown that both physical and chemical effects play an important role as inducers of transient synthesis of " heat-shock proteins " in yeast.[64] The heat shock response is an inducible protective system of all living cells.

This system simultaneously induces both heat shock proteins and an increased capacity for the cell to withstand potentially lethal temperatures (an increased thermotolerance). This has led to the suspicion that these two phenomena must be inexorable linked. However, analysis of heat shock protein function in Saccharomyces cerevisiae by molecular genetic techniques has revealed only a minority of the heat shock protein of this organism having appreciable influences on thermotolerance[99].

Pictures 3.5 (a) and 3.5 (b), show cells of *Saccharomyces cerevisiae* PLA 851 growing at 40 ^{o}C before and after incubation at 45 ^{o}C. As can be seen before incubation at high temperature, PLA 851 showed normal yeast cells characteristics, changing to a much smaller ellipsoidal and round form after exposition to 45 ^{o}C. Changes in the shape of the yeast cells may be related to different factors like amino acid concentration, starvation of nutrients and also membrane composition [29,38,39,46,94] .

As the reduction of the proportions of unsaturated fatty acids presently in the membrane can be an adaptative response on the part of an yeast to elevated temperatures [38], it may also result in the change in the shape of the yeast. These results imply that previous culture history has a significant effect on the response of cells to temperature.

PICTURE 3.5 a - Saccharomyces cerevisiae PLA 851 growing on 2 % initial sucrose medium at D = 0.10 h^{-1} and 40 ^{o}C, before incubation at 45 ^{o}C (Magnification X400).



PICTURE 3.5 b - Saccharomyces cerevisiae PLA 851 growing on 2 % initial sucrose medium at D = 0.10 h^{-1} and 40 ^{o}C, after incubation at 45 ^{o}C (Magnification X400).

3.6 - EFFECT OF INITIAL SUGAR CONCENTRATION ON THE OUTCOME OF CHEMOSTAT EXPERIMENTS WITH BIOMASS FEEDBACK AT 40 ^{o}C.

Ethanol production was tested for different initial sucrose concentration (2, 5, 10 and 20 %) at a variety of dilution rates (0.05; 0.10; 0.15; 0.20 and 0.25 h^{-1}) at 40 ^{o}C. The results can be seen in tables 3.6 (a), 3.6 (b), and 3.6 (c and d).

For a better comparison of the experiments, the B factor should have been kept constant. However, for operational reasons it was not possible. The ethanol yields, Yp (g of ethanol produced / g of sucrose) was determined following the equation:

$$Y = p / (s_o - s)$$

As can be seen from our results a marked improvement of ethanol yield was observed with an increase in the initial sugar concentration. The difference in yield improvements among the varying dilution rate at 2, 5 and 10 % of initial sucrose concentration was not so significant.

The very low yield and ethanol productivity observed at D = 0.25 h^{-1}, on 5 % of sucrose medium was probably due to the wash out of cells. However, at 20 % initial sucrose concentration there was an apparent improvement in the ethanol yield with increasing dilution rate.

The highest productivities were found at D = 0.25 h^{-1} for both 10 and 20 %, respectively (4.0 and 3.2 g/L.h). Nevertheless, the amount of residual sugar was very high. Apparently only 8 % of the input sugar has been consumed for 20 % initial sucrose at D = 0.25 h^{-1}.

A similar productivities were observed for 10 % initial sucrose concentration at D = 0.25 h^{-1}, where the highest yield value was found. However, the consumed sugar was about 39 % of the total sugar.

The maximum fermentation rate was obtained for an initial sugar concentration of 10 % and a non sugar concentration of 8 % . The authors found that the increasing in the sugar concentration leads to a linear increase in osmotic inhibition. It has been pointed out [14], that for an osmolality of 3 osmols, no growth is observed.

Dilution rate (h^{-1})	x (g/L)	s (g/L)	p (g/L)	Yp	P_{cont}
0.05	2.8	nd	10.6	0.53	0.3
0.10	2.7	nd	10.8	0.54	0.7
0.15	2.5	nd	10.5	0.52	1.1
0.20	2.2	nd	9.7	0.48	1.3
0.25	1.5	0.05	6.2	0.32	1.1

TABLE 3.6 a - The effect of dilution rate on Saccharomyces cerevisiae PLA 851 in 2 % sucrose medium at 40 ^{o}C in a Continuous culture with feedback of biomass.

Dilution rate (h^{-1})	x (g/L)	s (g/L)	p (g/L)	Yp	P_{cont}
0.05	3.4	nd	27.5	0.55	1.1
0.10	2.3	7.6	19.1	0.45	1.5
0.15	1.7	12.3	11.3	0.30	1.3
0.20	.97	37.5	8.1	0.64	1.0
0.25	.72	43.7	1.6	0.25	1.5

TABLE 3.6 b - The effect of dilution rate on Saccharomyces cerevisiae PLA 851 in 5 % sucrose medium at 40 ^{o}C in a Continuous culture with feedback of biomass.

Dilution rate (h^{-1})	x (g/L)	s (g/L)	p (g/L)	Yp	P_{cont}
0.05	2.3	42	32.1	0.55	1.2
0.10	2.1	48	24.2	0.46	1.7
0.15	1.5	64	19.0	0.52	2.3
0.20	1.2	72	10.5	0.37	1.6
0.25	1.9	61	22.4	0.57	4.0

TABLE 3.5 c - The effect of dilution rate on Saccharomyces cerevisiae PLA 851 in 10 % sucrose medium at 40 ^{o}C in a continuous culture with feedback of biomass.

Dilution rate (h^{-1})	x (g/L)	s (g/L)	p (g/L)	Yp	P_{cont}
0.05	2.2	155	28.9	0.64	0.9
0.10	2.1	162	24.7	0.65	1.5
0.15	1.8	173	16.9	0.62	2.0
0.20	2.1	179	11.6	0.55	3.2
0.25	2.0	187	7.0	0.53	3.2

TABLE 3.6 d - The effect of dilution rate on Saccharomyces cerevisiae PLA 851 in 20 % sucrose medium at 40 ^{o}C in a Continuous culture with feedback of biomass.

According to the workers [19,76,77,78], the limits of initial sucrose and non sugar concentration permitting optimal fermentation conditions are very narrow. When osmotic inhibition is present (1.5 < E_m < 3 osmols), sugar concentrations have an important impact but the best fermentation rates are obtained with high initial sugar concentration. see section 1.3.2.2.

The recycling of cells might minimize the effects of osmolarity inhibition at 20 % initial sugar concentration. Nevertheless, the highest ethanol productivity was achieved at 10 % initial sugar concentration.

Despite recent research, the production of ethanol by fermentation still remains more expensive than gasoline or ethanol produced by ethylene hydration. Although some improvements in ethanol productivities have been obtained using continuous culture techniques at high temperatures and microorganisms with the ability to ferment available and cheap substrates such as molasse and corn.

5 - REFERENCES

1 . Spencer, J.F.T. & Spencer, D.M. (1983) Ann. Rev. Microbiology 37: 121 - 142.

2 . Murtagh, J.E.(1986) Process Biochemistry 21: 61 - 65.

3 . Kosaric, N., Ng,D.C.M., Russell, I. and Stewart, G.S.(1980) Advances in Applied Microbiology 26: 147 - 227.

4 . Kosaric,N. Wieczorek,A., Cosentino, G.P., Magee,R.J. & Prenosil, J.E.(1983) Biotechnology 3: 257 - 385.

5 . Keim, C.R.(1983) Enzyme and Microbial Technology 5 : 103 - 114.

6 . Carioca, J.O.B., Arora H. L. and Khan, A.S. (1981) In: Advances in Biochemical Engineering 20: 153 - 161. Fiechter,A. Ed. Springer-Verlag, Berlin - GDR.

7 . Da Silva, J.E. (1983) In: Proceedings of the International Conference on the Commercial Applicati ons and Implications of Biotechnology 41 - 57.London

8 . Levinson, M. (1987) The Journal of Developing Areas 21: 245 - 258.

9 . Sorj, B. and Wilkinson, J. (1988) Biotechnology 6: 150 - 155.

10. Flinn, J. E. and Linpinsky, E. S. (1983) In : Proceedings The International Conference on the Commercial Applications and Implications of Biotechnology 523 - 538. London.

11. Standbury, P.F. and Whitaker, A. (1984) In : Principles of Fermentation Technology - Ed.Pergamon Press. Oxford, London.

12. Pirt, S. J., (1985) Principles of Microbe and Cell Cultivation. - (Blackwell Scientific Publications) London.

13. Monod, J. (1950) La Technique de Culture Continue Theorie et Applications. Ann. Inst. Pasteur 79: 390 - 410.

14. Novick, A. and Szilard, M. (1950) - Proc. Nat. Acad. Sciences 36 : 708 - 719.

15. Herbert, D., Elsworth, R. and Telling, R.C. (1956) The Journal of General Microbiology 14: 601 - 622.

16. Pirt, S. J. and Kurowsky, W. M. (1970) The Journal of General Microbiology 63: 357 - 366.

17. Pirt, S. J. (1969) Symposia of The Society for General Microbiology 19: 199 - 221.

18. Stokes, J. L. (1971) The Yeasts, 2 : 119 - 134. Ed. Academic Press - London.

19. Jones, R.P.,Greenfield, P. F.and Parment, N. (1981) Process Biochemistry 16: 42 - 49.

20. van Uden, N. (1984) Advances in Microbial Physiology 25: 195 - 251.

21. D'Amore,T. and Stewart, G. G. (1987) Enzyme and Microbial Technology 9: 322 - 330.

22. Wang, D.L.C., Cooney, C.T., Demain, A.I., Dunnil, P. Humphrey, A.E. and Lilly, H.D.(1979) Fermentation and Enzyme Technology. Eds. John Wiley and Sons, - New York.

23. Chye, J.H. and Goldstein, D.B. (1975) Journal of The Singapore National Academy of Science 4: 152 - 154.

24. Esener, A.A., Rosls, J.A. and Kossen, N.W.F. (1981) Biotechnology and Bioengineering 23: 1040 - 1045.

25. Ryu, Y.W. and Kwon, J.J. (1982) Korean Journal of Microbiology 20: 67 - 72.

26. Gilliland, R.B. (1981) Brewing Science 2: 1 - 60. Eds. J.R.A. Pollack, Academic Press - London.

27. van Uden, N. (1984) Advances in Microbial Physiology 25: 195 - 251.

28. Lund, A. (1954) Studies on the Ecology of Yeasts, Munksgaard - Copenhagen.

29. Watson, K. (1987) The Yeasts 2: 41 - 71. Eds. A.H. Rose and J.S. Harrison. Academic Press - London.

30. Kogut, M.(1980) Trends in Biochemical Sciences 5: 15 - 18.

31. Russel, N. J. (1984) Trends in Biotechnology 9: 100 - 112.

32. Pfisterer, E., Hancock, I. and Garrison, I. (1977) Journal of American Society of Brewing Chemistry 35: 49 - 54.

33. Navarro, J.M. and Durand, G. (1978) Annales de Microbiologie (Institute Pasteur) 129 B: 215 - 224.

34. Novak, M., Strehaiano, P., Moreno, M. and Goma, G. Biotechnology and Bioengineering 23: 201 - 211.

35. Pina, A., Calderon, I.L. and Benitez, T. (1986) Appl. and Environmental Microbiology 51: 995 - 1003.

36. Sa-Correia, I. and van Uden, N. (1982) Biotechnology Letters, 4: 805 - 808.

37. Sa-correia, I. and van Uden, N. (1983) Biotechnology and Bioengineering 25: 1665 - 1667.

38. Thomas, S.D., Hossack, J.A. and Rose, A. H. (1978) Archives of Microbiology 117: 239 - 245.

39. Arthur, H. and Watson, K. (1976) The Journal of Bacteriology 128: 56 - 68.

40. Leao, C. and van Uden, N. (1982 a) Biotechnology and Bioengineering 24: 1581 - 1583.

41. Bottema, C.D.K., McLean-Bowen, C.A. and Parks, L.W. (1983) Biochimica et Biophysica Acta 734: 235 - 248.

42. Lodder, J. (1984) The Yeasts - A taxonomic study. North - Holland Publishing, Amsterdam.

43. Contreras, R., Delgano, R., Fernadez, N.R. and Mendez, N. (1982) Review Latin - American Microbiology 24: 167 - 171.

44. Esser, K. and Schmidt, U. (1982) Process Biochemistry 7: 46 - 49.

45. Slapack, G.E., Russell, I. and Stewart, G.G. (1987) Uniscience Series, CRC Press. Boca Raton - Florida.

46. Watson, K., Arthur, H. and Blakey, M. (1980) Journal of Bacteriology 143: 693 - 695.

47. Hughes, D.B., Tudroszen, N.J. and Moye, C.J. (1984) Biotechnology Letters 6: 1 - 6.

48. Rosa, M. F., Sa-Correia, I. and Novais, J. M.(1987a) Biotechnology Letters 9: 441 - 444.

49. Krouwel, P.G. and Braber, L.(1979) Biotechnology Letters, 1: 403 - 408.

50. D'Amore, T., Panchal, C.J., Russell, I. and Stewart, G.G. (1988) Journal of Industrial Microbiology 2: 365 - 372.

51. D'Amore, T., Celloto, G., Russell, I. and Stewart, G.G. (1989) Enzyme and Microbial Technology 11: 411 - 416.

52. Kosaric, N., Ng, D. C. M., Russell, I. and Stewart, G.G. (1980) Advances in Appl. Microbiology 26: 147 - 227.

53. Rose, H.A. and Beaven, M.J. (1981) In: Trends in The Biology of Fermentation for Fuel and Chemicals 513 - 531.

54. Brown, S.W., Oliver, S.G., Harrison, D.E.F. and Riguelato, R. C. (1981) European Journal of Applied Microbiology and Biotechnology 11: 151 - 155.

55. Holzberg, I., Finn, R. K. and Steinkraus, K. H. (1967) Biotechnology and Bioengineering 9: 413 - 427.

56. Aiba, S., Shoda, M. and Nagatani, M. (1968) Biotechnology and Bioengineering 10: 845 - 864.

57. Sa-Correia, I. and van Uden, N. (1985) Biotechnology and Bioengineering 18: 301 - 303.

58. Leao, C. and van Uden, N. (1982 b) Biotechnology and Bioengineering 24: 1601 - 1604.

59. Leao, C. and van Uden, N. (1983) Biotechnology and Bioengineering 25: 2085 - 2090.

60. Saita, M. and Slaughter, J.C. (1984) Enzyme and Microbial and Technology 6: 375 - 378.

61. Loureiro-Dias, M. C. and Peinado, J. M. (1982) Biotechnology Letters 4 : 721 - 724.

62. Rose, A. H. (1980) In: Biology and Activity of Yeast, 103 - 121. Eds. Skinner,F.A., Passmore, S.M. and Davenport R. R. - Academic Press , London.

63. Gray, W. D. (1948) Journal of Bacteriology 55: 53 - 59.

64. Millar, D. G., Griffiths-Smith, E. Algar and Scopes, R.K.(1982) Biotechnology Letters 4: 601 - 604.

65. Luria, S. E. (1975) Scientific American 233: 32.

66. Ingram, L. O. and Buttke, T. M. (1984) Advances in Microbial Physiology 25: 253 - 300.

67. Romano, A. H. (1986) Trends in Biotechnology 4: 207 - 213.

68. Dekker, R. F. H. (1986) Biotechnology Letters 28: 605 - 608.

69. Thomas, D. S., Hossacks, J. A. and Rose, A. H. (1978) Archives of Microbiology 117: 239 - 245.

70. Casey, G. P. and Ingledew, W. M. (1986) C R C Critical Reviews in Microbiology 13: 219 - 280.

71. Brown, S. W. and Oliver, S. G. (1982) European Journal Appl. Microbiology and Biotechnol. 16: 119 - 122.

72. Jones, R.P. and Greenfield, P.F. (1984) Biotechnology Letters 6: 471 - 476.

73. Walker-Caprioglio, H.M., Rodriguez, R.J. and Parks, L.W.(1985) Applied and Environmental Microbiology 50: 685 - 689.

74. Carruthers, A. and Melchior, D. L. (1986) Trends in Biochemical Sciences 11: 331 - 335.

75. Converti, A., Perego, P., Lodi, A., Parisi, F. and Borghi, M. (1984) Biotechnol.and Bioengineering 27: 1108 - 1114.

76. Gray, W. D. (1946) Journal of Bacteriology 52: 703 - 709.

77. Panchal, C. L. and Stewart, G. G. (1980) Journal of the Institute of Brewing 86: 207 - 210.

78. Maiorella, B., Wilke, C. R. and Blanch, W. (1981) Advances in Biochemical Engineering 20: 43 - 92.

79. Desari, G., Roddick, F., Connor, M.A. and Pamment, N. B.(1983) Biotechnology Letters 5: 715 - 720.

80. Thomas,, S. D. and Rose, A. H. (1979) Archives of Microbiology 122: 49 - 55.

81. Desari, G., Keshavarz, E., Connor, M.A. and Pamment, N.B. (1985) Biotechnology Letters 7: 541 - 546.

82. Ingram, L.O. (1986) Trends in Biotechnology 2: 40 - 44.

83. Holzer, H. (1976) Trends in Biochemical Sciences 1: 178 - 181.

84. Barford, J. P. and Hall, R.J.(1979) The Journal of General Microbiology 114, 267 - 275.

85. Ryu, D. D .Y., Kim, Y. J. and Kim, J. H. (1984) Biotechnology and Bioengineering 26: 12 - 16.

86. D'Amore, T., Panchal, C.J., Russell, I. and Stewart, G.G. (1988) Journal of Industrial Microbiology 2: 365 - 372.

87. D'Amore, T., Panchal, C.J., Russell, I. and Stewart, G.G. (1990) Critical Reviews in Biotechnology 9: 287 - 304.

88. Springham,D.G. (1991) In : Biotechnology - The Science and the Business. Chapter 16, 249 - 295. Eds. V. Moses and R. E. Cape. Harwood Acad Publishers. London.

89. Cysewski, G. R. and Wilke, C. R. (1976) Biotechnology Bioengineering 20: 1421 - 1423.

90. Neidleman, S. L. (1991) In: Biotechnology the Science and the Business. Chapter 17, 297 - 310. Eds. V. Moses and R.E. Cape. Harwood Acad Publishers. London.

91. Ledingham, W. M. and Kinghorn, J. R. (1985) Technical Report to NATT/CRPAA - Maceio - Alagoas, Brazil.

92. Ledingham, W, M. (1988) Technical report to NATT/CRPAA Maceio - Alagoas, Brazil.

93. Harder, W. (1987) In : Proc. 4th Eur. Congress on Biotechnology 4: 109 - 120.

94. Borzani, W. (1986) Biotechnology Letters 8: 187 - 190.

95. Porro, D., Martegani, E., Ranzi, M. B. and Alberghina, L. (1988) Biotechnology and Bioengineering 32: 411-417.

96. Lee, G. M., Song, K. B., Rhee, S. K. and Han, M. H. (1986) Biotechnology Letters 8: 385 - 390.

97. Mishra, Prashant. and Prasad, Rajendra. (1988) Journal of General Microbiology 134: 3205 - 3211.

98. Hall, M.J., Dickinson,S.D., Pritchard, R. and Evans, J.I. (1973) Prog. Ind. Microbiol. 12: 171 - 234.

99. Piper, P.W. (1993) FEMS - Microbiology Rev. 11: 339-356.

100. Gilson, Christopher D. and Thomas, A. (1995) J. Chem. Tech. and Biotechnology (1995) 62: 38 - 45.

101. Jinghong Li, P. James McLellan and Daugulis, A.J. (1995) Biotechnology Letters 17: 321 - 326.

102. Ramos,M.T. and Madeira-Lopes, A. (1990) Biotechnology Letters 12 : 229 - 234.

103. Carvalho, J.C.M., Aquarone, E., Sato, E., Brazzach, M.L., Almeida, K.A. and Borzani, W (1990) Biotechnology Letters 12: 777 - 782.

104. Laluce, C., Abud, C.L., Greenhalf, W., Sanches - Peres, M.F. (1990) Biotechnology Letters 15: 609 - 614.

105. Hsioao, Tzu-Yin and Glatz, C.E. (1994) Biotechnology and Bioengineering 44: 1228 - 1234.

106. Parekh, S.R. and Cheryan, M. (1994) Enzyme Microb. Technol. 16: 104 - 109.

107. San Martin, R., Bushell, D., Leak, D.J. and Hartley, B.S. (1994) Biotechnology and Bioengineering 44: 21 - 28.

108. Maia, A.B.R.A. and Nelson, D.L. (1993) Biotechnology and Bioengineering 41: 361 - 369.

109. Moser, A. (1990) Biotechnology and Bioengineering 37: 1054 - 1065.